# BIOPHYSICS

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# БИОФИЗИКА

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VOL
5
196

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OL.
5
960

VOL.
5
196

# CONTENTS OF VOLUME 5, 1960

## NUMBER 1


D. M. SPITKOVSKII, P. I. TSEITLIN and V. S. TONGUR: Some effects caused by the two configurations of DNA . . . . . 1

A. G. PASYNSKII: The role of matrix structures in replication . . . . . 12

I. Ye. EL'PINER and A. V. SOKOL'SKAYA: Oxidation processes of biologically-active substances in a field of ultrasonic waves . . . . . 18

M. S. LEVINSON and B. G. KOVROV: A photoelectric study of the kinetics of oxyhaemoglobin cleavage under the action of alkalis and acids . . . . . 25

L. G. VIEZE and G. M. FRANK: Structural mobility and auto-regulation of cell processes 31

R. G. LYUDKOVSKAYA and L. P. KAYUSHIN: Action of ultra-violet radiation on single nerve fibres . . . . . 38

I. M. VASIL'EV and B. G. ZHUKOV; The effect of fractionated doses of X-irradiation on the capacity of plants to resume growth . . . . . 45

L. P. BRESLAVETS: Effect of chronic irradiation on dried rye seeds . . . . . 48

I. D. VINOGRADOV: The radiosensitivity and embryogenesis of *Ascaris suis* ova . . . . 54

G. A. VOLKOV and G. R. RIK: $\alpha$-ray dose rate near an interface between different media 60

Methods and instruments

M. M. ZAALISHVILI, F. O. SHRAIBMAN and A. R. EGIAZAROVA: An automatic system for determining the diffusion coefficient . . . . . 69

V. A. STRUCHKOV: An automatic fraction collector . . . . . 79

A. I. SHAPOVALOV: Double-barrelled microelectrodes for intracellular measurement of biopotentials . . . . . 83

Practical notes

L. P. BRESLAVETS, N. M. BEREZINA, G. I. SHCHIBRYA, M. L. ROMANCHIKOVA, V. A. YAZYKOVA and Z. F. MILESHKO: Increase in the yield of radishes and carrots by X- or $\gamma$-irradiation of the seeds before sowing . . . . . 86

V. V. KHVOSTOVA and S. A. VALEVA: The application of ionizing radiation in plant selection . . . . . 87

Letters to the Editor

T. G MAMEDOV: The effect of irradiation on the permeability of radicles of *Vicia faba* 92

V. N. KOROGODIN and N. V. LUCHNIK: Nature of primary changes in cell radiation damage . . . . . 96

D. M. SPITKOVSKII: Determination of some molecular parameters of ribonucleic acid in solution . . . . . 100

Discussion

A. S. TROSHIN: Concerning an article by L. M. Chailakhian — Modern concepts of the nature of the resting potential . . . . . 104

L. V. CHKHAIDZE: Basic problems in the study of co-ordination of voluntary movements in man from the viewpoint of biophysics . . . . . 112

Book reviews

B. N. VEPRINTSEV: Die Muskeltätigkeit . . . . . 118

V. N. CHERNIGOVSKII: Local reaction and diffuse excitation of protoplasm . . . . . 121

Science news

A. N. TERENIN: Energy transfer with special reference to biological problems . . . . . 123

V. G. SAMSONOVA: Electrophysiology and biophysics of the sensory organs at the XXI International Congress of Physiologists . . . . . 134



VOL.
5
1960

## NUMBER 2


N. N. BUBNOV, A. A. KRASNOVSKII, A. V. UMRIKHINA, V. F. TSEPALOV and V. IA. SHLIAPINTOKH: Electron spin resonance spectra observed on illumination of plant leaves and in the photo-reduction of chlorophyll and its analogues . . . . . 145

YU. A. VLADIMIROV and F. F. LITVIN: Long-lived phosphorescence of aromatic amino-acids and proteins at low temperatures . . . . . 151

N. N. KOKINA: Ionic interrelations and the role of potassium in the rhythmic variations in the intracellular potential of infusoria . . . . . 159

I. A. ARSHAVSKII: Perielectrotonus as the basis and mechanism of production of excitation and inhibition (Biophysical mechanism of autovibratory processes) . . . . . 168

V. D. GLEZER, R. G. MANGUSHEV and A. B. ATLAVIN: Role of the nervous component in dark adaptation . . . . . 177

A. L. YARBUS: Perception of images of variable brightness fixed with respect to the retina of the eye . . . . . 183

B. KH. GUREVICH: Conditioned fixation reflexes and certain aspects of the cybernetics of eye movements in man . . . . . 188

A. A. ZOTIKOV: Effect of ionizing radiation on the luminous bacteria *B. issatchenkoi* (Radio-biological problem of the time factor and of recovery) . . . . . 197

YE. V. MOISEENKO: Nucleic acid content and radiosensitivity of *Escherichia coli* . . . . 203

I. I. GITEL'ZON and I. A. TERSKOV: The "aftereffect reaction" in irradiated erythrocytes 207

V. I. KOROGODIN and T. G. MAMEDOV: Influence of irradiated plant seedlings on the growth of unirradiated seedlings . . . . . 213

I. M. VASIL'EV and B. G. ZHUKOV: Reaction of the plant to injury after irradiation . . . 217

YE. V. BURLAKOVA and O. R. KOL'S: Study of the electrical conductivity of the liver of irradiated animals . . . . . 223

YU. I. MOSKALEV: Role of the time factor in damage due to radioactive isotopes . . . . . 231

G. B. RADZIYEVSKII: Measurement of absorbed dose in an inhomogenous field of radiation with an extrapolation chamber with diaphragm . . . . . 238

Methods and apparatus

A. M. KONONENKO and V. A. PETROV: Certain aspects of the dosimetry of distributed sources of $\beta$-radiation . . . . . 250

YU. YE. MOSKALENKO: Application of centimetre radio waves for non-contact recording of changes in volume of biological specimens . . . . . 259

F. R. VINOGRAD-FINKEL', L. L. RAZUMOVA and S. N. KUDRYASHOVA: Application of X-ray diffraction to investigations on frozen blood . . . . . 264

Letters to the Editor

G. K. MOROZOVA and L. A. BLYUMENFEL'D: Effect of oxygen on electron spin resonance spectra of lyophilized tissues . . . . . 273

V. YU. URBAKH: Some observations on the biophysical theory of memory . . . . . 276

YU. A. KRIGER and I. M. PARKHOMENKO: Problem of the protective action of thiourea 278

I. YE. EL'PINER, I. M. DRIZE and I. M. FAIKIN: Ultrasonic images of the organs and tissues of the animal organism . . . . . 281

Biophysics in practice

S. A. VALEVA: Data on the radiosensitivity of agricultural crops . . . . . 284


## NUMBER 3


On the ninetieth anniversary of the birth of the great Lenin . . . . . 291

YU. N. POLYANSKII: Characteristics of ageing of a gel-solution of actomyosin . . . . . 292

V. K. TKACH and L. A. FRENKEL': Study of the stability of proteins by the method of non-steady heat exchange of their solutions in a high frequency field . . . . . 295


VOL
5
196

G. A. KURELLA: The sorption theory of cell permeability and the pre-existence of resting potentials . . . . . 303
A. I. SHAPOVALOV: Effect of temperature on the fast and slow potential of the rhythmically active muscle fibre, with intracellular recording . . . . . 314
L. P. KAYUSHIN, R. G. LYUDKOVSKAYA and I. P. SHMELEV: Ultra-violet absorption by the *Sepia* giant axon in states of rest and excitation . . . . . 323
A. L. BYZOV: Analysis of distribution of potential and currents within the retina on light stimulation — II. Passive distribution of potentials and current within the retina as in a volume conductor . . . . . 328
A. L. YARBUS: Perception of images of great brightness, motionless with respect to the retina of the eye . . . . . 338
G. A. MAZOKHIN-PORSHNYAKOV: Colorimetric study of the properties of colour vision of insects as exemplified by the house fly . . . . . 340
N. A. GORLANOV: Participation of electric potential differences in the translocation of phosphorus ($^{32}P$) in the roots of the onion . . . . . 350
S. V. TAGEEVA and A. B. BRANDT: Study of the optical properties of leaves in relation to the angle of incidence of light . . . . . 354
Ye. N. MOSYAGINA: Determination of the mean life span of erythrocytes entering and circulating in the blood . . . . . 365
I. Sh. MOREINIS: Problem of biomechanical asymmetry and its correction on application of prothesis . . . . . 375
L. Kh. EIDUS and Ye. E. GANASSI: Existence of several types of latent damage in irradiated myosin molecules . . . . . 384
I. K. KOLOMIITSEVA: Formation of stable organic peroxides in the living body as a result of ionising radiation . . . . . 389
N. B. STRAZHEVSKAYA: Effect of ionising radiation on protein metabolism in nuclei, mitochondria and ergastoplasm of the plant organism . . . . . 397
G. K. GURTOVOI and Ye. O. BURDYANSKAYA: Exact dose X-irradiation of various regions of the head and visual sensations — X-ray location method of study of the reactivity of the central nervous system . . . . . 406
S. A. VALEVA: Effect of $\gamma$-rays and neutrons on dried seeds in agricultural plants . . . . . 415
Methods and instruments
V. L. BOROVYAGIN and M. G. KUPTSOV: Some improvements in the UEM-100 electron microscope for the investigation of biological specimens . . . . . 420
M. Ya. KHODAS: Method of fluorescence microscopy of peripheral blood . . . . . 425
G. A. KURELLA and G. A. POPOV: Determination of pH with an antimony microelectrode 430
L. S. GOLD'IN and V. S. PETROV: Method of embedding histological material in methacrylate . . . . . 432
Letters to the Editor
K. M. L'VOV, I. P. KAYUSHIN, and R. G. LYUDKOVSKAYA: Relation between the elastic properties of nerve fibre and certain functional characteristics . . . . . 437
Current events
International symposium on electro-retinography held in Czechoslovakia . . . . . 440

## NUMBER 4

Yu. A. VLADIMIROV and E. A. BURSHTEIN: Luminescence spectra of aromatic amino acids and proteins . . . . . 445
P. I. TSEITLIN, D. M. SPITKOVSKII and N. P. RYABCHENKO: Relation between molecular morphology of DNA macromolecules and their radiosensitivity . . . . . 454
V. K. TKACH: Certain features of the process of disturbances in the structural stability of serum proteins in malignant tumours . . . . . 459

L. P. KUZNETSOVA: Electrophysiological study of the threshold sensitivity of the dark-adapted eye of the frog and the influence on it of red and blue light . . . . . 466
G. V. GLEKIN and G. M. ERDMAN: Discrimination of a useful signal by the auditory analyser 474
S. N. GOL'DBURT: Adequatometry of fast developing auditory adaptation (masking of short tones by long sinusoidal tones) . . . . . 482
B. L. KARPMAN: Analysis of the kinematics of the human body on cardiac contraction (vectoballistocardiography) . . . . . 492
A. P. DUBROV: Photoreactivation of plant cells . . . . . 501
SH. D. KHAN-MAGOMETOVA, A. V. GUTKINA, M. N. MEISEL', L. S. AGROSKIN and N. V. KOROLEV: Ultra-violet fluorescence of certain animal organs and its change on irradiation . . . . . 509
L. M. KRIUKOVA and A. M. KUZIN: Distance effect of ionising radiation on plants . . . 513
S. YA. RAPOPORT and S. M. GASANOVA: Mechanism of early radiation damage to the bone marrow . . . . . 517
D. D. ROMASHOV, K. A. GOLOVINSKAYA, V. N. BELYAEVA, E. D. BAKULINA, G. L. POKROVSKAYA, and N. B. CHERFAS: Diploid radiation gynogenesis in fish 524
I. B. BYCHKOVSKAYA and G. K. OCHINSKAYA: Protective effect ofhypoxia at various radiation doses . . . . . 532
G. K. GURTOVOI and TE. O. BURDYANSKAYA: Threshold reactivity of various regions of the human retina to X-irradiation . . . . . 538
Methods and apparatus
B. M. ISAEV: Physical bases of radiobiological experiments with neutron fluxes . . . . . 543
V. A. IL'YANOK: A technique for studying high frequency potentials of the e.e.g. . . . . 555
A. P. SHAKHNOVICH: Principle of pupil scanning . . . . . 561
A. B. BRANDT: Automatic device for switching electric installations on and off . . . . . 565
Letters to the Editor
L. M. TSOFINA: Effect of bromine ions on the activity of the frog retina . . . . . 567
T. S. TRINCHER: Physical significance of the dose-coefficient on thermal treatment of blood 571
Biophysics in practice
I. G. KOSTIN: Experience with the use of microdoses of $\gamma$-radiation in poultry breeding 572
Discussion
A. K. PRITS: Thermodynamics of irreversible processes and excitation theory . . . . . 575
Current events
G. A. DVORKIN: Conference on biophysics of erythrocytes . . . . . 579
Reviews
N. M. LIVENTSEV: "Essentials of biological and medical physics". By R. Stacy, D. Williams, R. Worden and R. McMorris . . . . . 582

## NUMBER 5

A. M. TONGUR and A. G. PASYNSKII: Changes in the surface properties of irradiated DNP and DNA . . . . . 587
L. KH. EIDUS, YE. E. GANASSI: Analysis of the effect of the chief factors changing radiosensitivity . . . . . 594
A. M. KUZIN and K. S. TRINCHER: Change in erythrocyte radiosensitivity . . . . . 605
YU. A. KRIGER and I. M. PARKHOMENKO: Change in the properties of erythrocytes when exposed to sub-haemolytic doses of gamma-radiation . . . . . 611
M. D. POMERANTSEVA: Remote effect of radiation on mouse testes . . . . . 616
V. MAL'TS: Formation of organic peroxides in the liver of rats irradiated with ionizing rays 619
YE. V. BURLAKOVA and I. M. PARKHOMENKO: Investigation of the electrical conductivity and autolysis of the hepatic tissue of irradiated animals . . . . . 625
V. V. SAKHAROV, V. V. MANSUROVA, R. N. PLATONOVA and V. K. SHCHERBAKOV:

VOL.
5
196

Detection of physiological protection against ionizing radiations in autotetraploids of seed buckwheat . . . . . 632
A. M. KUZIN, N. M. BEREZINA and O. N. SHLYKOVA: Relation between dose rate and the radiobiological effects in plants . . . . . 643
I. M. VASIL'EV, B. G. ZHUKOV and T. S. SPASSKAYA: Intensification of effect of X-irradiation on seeds during storage . . . . . 646
I. E. EL'PINER and O. M. ZORINA: Effect of ultrasonic radiation on ribonuclease . . . . 649
L. V. VOZYSHAEVA and L. A. BLYUMENFEL'D: The effect of ionized side groups on the magnetic properties of RNA . . . . . 656
A. L. ZAIDES: Problem of the polyphase nature of collagen . . . . . 659
P. G. KOSTYUK and A. I. SHAPOVALOV: Relation between electrical polarization and rhythmical activity of striated muscle fibre . . . . . 664
V. Z. OSHEROVICH: Determination of the angular displacements of the members of the upper limb from the co-ordinates of its joints . . . . . 674
V. B. YEVSTIGNEYEV and V. A. GAVRILOVA: Ability of chlorophyll to photosensitize redox reactions in the absorbed state . . . . . 679
L. A. MILYUTIN: A mechanism of electrical asymmetry in tissues . . . . . 689
Methods and instruments
A. I. POLIVODA and A. A. MIKHAILOVA: Study of the electrical constants of rat liver tissue 693
M. I. MEKSHENKOV and A. P. ANDREITSEV: A method of photoelectric compensation 698
E. B. BABSKY, A. B. VENEDIKTOV, V. L. KARPMAN and M. I. TISHCHENKO: The dynamocardiograph . . . . . 703
I. A. UTINA: Investigation of the activity of bipolars of two types by the method of ultra-violet cytophotometry . . . . . 710
A. V. BIBERGAL': $\gamma$-unit for chronic irradiation in radiobiological experiments . . . . . 713
Ye. Ye. KOVALEV and D. P. OSANOV: Fraction of radiation emerging from a hollow cylindrical source filled with absorbing substance . . . . . 716
Letters to the Editor
A. A. ZOTIKOV: Dosimetry of radiation of pulsed X-ray tubes with autoelectron emission, and the possibility of their use in radiobiology . . . . . 720
I. K. KOLOMIITSEVA, K. M. L'VOV and L. P. KAYUSHIN:Determination of free radicals in tissues of rats inoculated with sarcoma 45 . . . . . 723
A. V. S. YUSIPOV: Problem of vitamin C in radiation sickness . . . . . 724
Discussion
A. K. PRITS: Stationary states and the thermodynamic theory of excitation of living tissues . . 726

## NUMBER 6

SHEN PEI-KEN, L. A. BLYUMENFEL'D and A. E. KALMANSON: Effect of denaturation and complexing with proteins on the magnetic properties of nucleic acids . . . . . 733
G. B. LINKOVSKII: Stability memory of the brain treated as a system of autonomic "memory" 745
R. G. LYUDKOVSKAYA and L. P. KAYUSHIN: Photodynamic effect of various fluorescent dyes on the excitable giant axon . . . . . 754
L. A. CHISTOVICH: Perception of sound sequence . . . . . 762
P. O. MAKAROV: Effect of very strong optical stimulation on the visual, auditory and skin analysers in Man . . . . . 769
T. M. KROL': Relation between absolute light threshold and the rate of rise of a stimulus lasting less than the "useful" time . . . . . 778
P. O. MAKAROV and T. M. KROL': Significance of the factors of intensity, duration and area in determining the excitability of the human visual analyser . . . . . 784
G. A. MAZOKHIN-PORSHNYAKOV: The system of colour vision of the fly *Calliphora* 790
P. N. IVANOV, A. G. KUZNETSOV, V. B. MALKIN and Ye. O. POPOVA: Decompression phenomena in the human body in conditions of extremely low atmospheric pressure . . . 797

M. S. USPENSKAYA: Spectrometric of radioisotope study of preparations DNA isolated from the rat liver after exposure to $^{90}Sr$ . . . . . 803
A. M. KUZIN and V. A. KOPYLOV: Disturbance of oxidative-reduction processes in plant tissues under the influence of ionizing radiation . . . . . 810
A. D. STRZHIZHOVSKII: Local reaction of tissue to irradiation . . . . . 814
Methods and instruments
L. B. KAMINIR and L. L. LITINSKAYA: Cytometric analysis of blood by electronic methods. 820
L. N. ZHINKIN and A. A. ZAVARZIN: Study by the method of autoradiography of the incorporation of S-labelled cysteamine, methionine and sodium sulphate . . . . . 830
K. K. AGLINTSEV: Function of recovery on irradiation of organisms . . . . . 839
Letters to the editors
G. P. GEORGIYEV and V. A. STRUCHKOV: Degree of polymerization of DNA of animal origin . . . . . 845
I. A. UTINA, N. V. NECHAYEVA and V. YA. BRODSKII: DNA in ganglion cells of frog retina in the dark and on illumination with constant and flickering light . . . . . 849
V. R. PROTASOV and M. I. ARONOV: Biological significance of the sounds of certain Black Sea fish . . . . . 851
N. I. SHAPIRO and N. V. BELITSINA: Chemical structure of streptomycin and change in frequency of nuclear damage of irradiated mammalian cells . . . . . 853
Discussion
V. L. VOZNESENSKII: An error in measuring the intensity of photosynthesis by conductimetric method . . . . . 856
Current events
K. S. TRINCHER and V. I. TOKARSKAYA: Primary and initial mechanisms of biological action of nuclear radiations . . . . . 860
Review
KH. S. KOSHTOYANTS: J. Bureš, M. Petráň and J. Zachar: "Electrophysiological methods in biological research". Published by the Czechoslovak Academy of Sciences. Prague 1960. 864

VOL
5
196

VOL.
5
1960

VOL
5
196

# SOME EFFECTS CAUSED BY THE TWO CONFIGURATIONS OF DNA*

D. M. SPITKOVSKII, P. I. TSEITLIN and V. S. TONGUR

Institute of Experimental Biology, U.S.S.R. Academy of Medical Sciences, Moscow

(*Received* 15 *April* 1959)

VOL. 5 1960

FREE DNA differs in molecular shape from DNA bound to protein [1–4]; in the latter form the DNA molecule takes up a fairly compact shape. The viscosity is thereby reduced. It is found that almost any agent changes the shape [1] (here we have in mind agents whose effects are not such as to reduce the molecular weight or to denature the DNA).

In this paper we consider the compact form, and seek to establish whether that shape is inherent in the molecule, or whether it is produced by the agents. Various configuration types (*A*, *B*, *C*, $C_1$, $C_2$, etc) have been described (we deal with these below), but these have merely been reported, and no attempt has been made to relate the changes in configuration to the conditions to which the DNA has been subjected; even the relation of one type to another is not known. Chargaff [5] says that "a nucleoprotein is a unity, and contains a multiplicity of possible structures". The same may, no doubt, be said of DNA; but DNA takes part in biological processes (hence its biological importance), and so it may be possible to differentiate various states, each of which corresponds to some exact configuration responsible for a biological reaction.

Two distinct forms are known, namely crystalline *A* and paracrystalline *B* [6]. The *A* form is found at about 75 per cent relative humidity; it contains about 30 per cent water. At higher humidities the molecule increases in length by about 30 per cent and the compound goes over to the less highly ordered *B* form. The transition is reversible, so the relation between the structures must be simple. In the *B* form the spiral has a repeat distance of 34 Å, with 10 nucleotides in that distance; the bases lie at right angles to the axis. In the A form the bases deviate from the perpendicular position by about 20°, which allows the chain to contract about 30 per cent; the axial projection of each nucleotide is then only 2·55 Å, which corresponds to a repeat distance of 28 Å, with 11 nucleotides per turn. Fig. 1 in reference [7] shows the *B* form schematically. The tables [7] assign exactly the same coordinates for the atoms and groups to both forms. The two forms differ in density [9] and in their infrared absorption bands [8].

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Sadron [10] and Pouyet and Weill [11] claim that the $C_1$ and $C_2$ forms exist in solution on the basis of the $[\eta] = f(M)$ curves ($\eta$ is viscosity, $M$ is molecular weight). They consider that the type $C_1$ molecules are more unsymmetrical than the type $C_2$ ones.

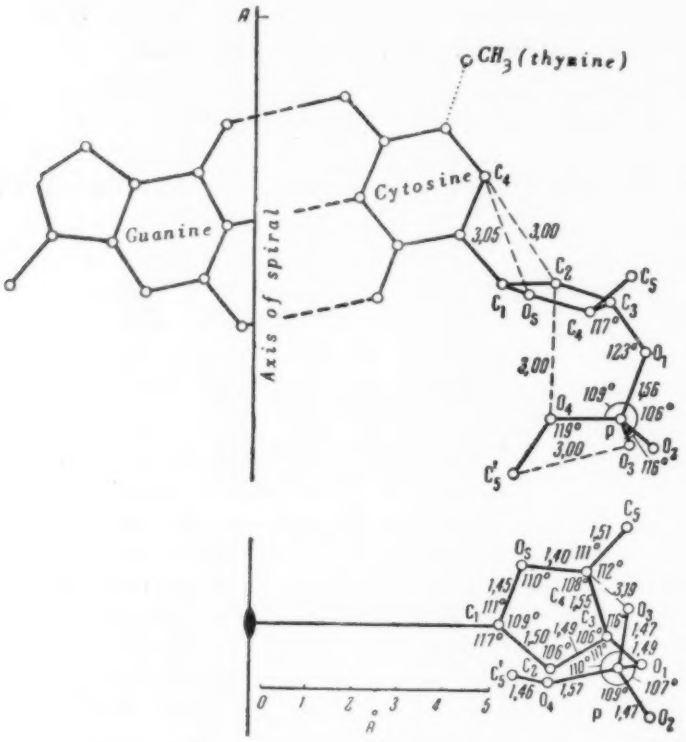


FIG. 1. The *B* form of DNA. The valence angles and bond lengths are shown.

The few published results show that DNA exists in several distinct configurations.

Various agents cause the viscosity of DNA solutions to fall to a half; if we treat the molecule as an ellipsoid (although other forms are quite possible) it is quite clear why a 30 per cent change in length can cause this effect (Fig. 2). On the other hand, the change from the *B* to the *A* form is also accompanied by a 30 per cent change in asymmetry. We shall show below that the $C_1$ and $C_2$ forms show similar differences.

The changes produced in various ways (by protein, by the *A*–*B* transition, by the $C_1$–$C_2$ transition) are the same in magnitude, and so we conclude that a single cause is responsible. In consequence, in our view, one of the forms is always the crystalline *A* form; this is confirmed by the steric correspondance between the *A* configuration of DNA on the one hand and the $\alpha$ configuration of the protein on the other [12]. The constancy of the *A* form is also to be expected from the fact that any simple long-chain substance tends to crystallize as one of its possible rotation isomers, and so

does a polymer [14]. A purely phenomenological analogy can be drawn in the case of gutta percha. This substance exists [15] in two crystalline forms, which correspond to the less and more extended rotation isomers. The first is stable; the second is metastable. The more extended configuration is adopted when the stable form is stretched [16].

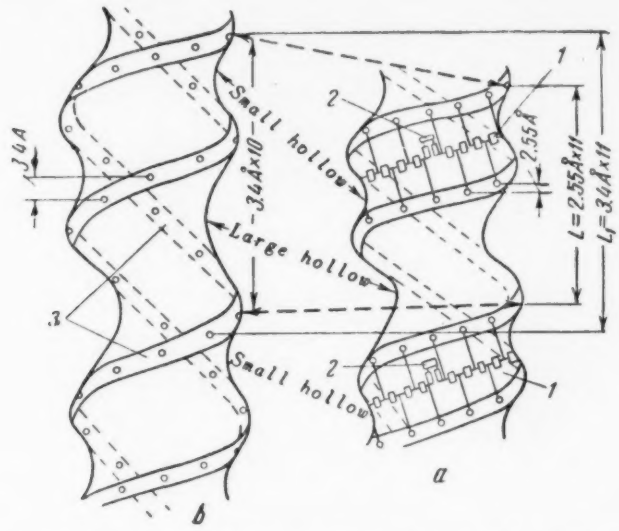


FIG. 2. The $A \rightleftarrows B$ transitions of DNA: (a) The $A$ form of DNA in DNP; (b) the $B$ form: (1) polypeptide chain with side groups, (2) possible amino-acid loops; (3) phosphate groups of DNA.

A natural inference is that DNA goes over to its most stable ($A$) form when any mild agent acts on it. The reasons for this are dealt with below. One of the reasons (if not the main reason) for the crystallization is that the chains are somewhat flexible. Florey [17] has shown that the crystalline state is the most stable one if the chain is reasonably rigid. Simple (monomeric) substances crystallize only on account of the forces between molecules, but a parallel ordered array of polymer chains may (in principle) be stable only if the chains are rigid. On the other hand, the chains presumably become less flexible as they become shorter, so we would expect that, as the molecular weight of DNA falls, a point would eventually be reached such that the DNA could exist only in the compact $A$ form (the molecule is in any case rather rigid). Some calculations illustrate this expectation.

One of us [12] has made use of the results that Florey and Fox's formula corresponds in fact with the behaviour of DNA, and that the segment of the molecule increases with the molecular weight. This knowledge about the segment enables us to calculate the quantity $\beta^3 K_1$ appearing in the equation

$$[\eta] = \beta^3 K_1 M^{1.2} \qquad (1)$$

(in which $\beta^3$ is the volume expansion coefficient and $K_1$ is a constant) for any given molecular weight.

TABLE 1. VALUES OF $K$ IN THE EQUATION $[\eta] = KM$

| $M$ ($\times 10^{-6}$) | $[\eta]$ (100 cm$^3$/g) | $K$ ($\times 10^6$) | |
|---|---|---|---|
| | | *B* form | *A* form |
| 0·2 | | 9·0 | 4·08 |
| 0·3 | 1·77 | 9·15 | 4·17 |
| 0·45 | 3·12 | 11·2 | 5·08 |
| 0·7 | 5·35 | 7·5 | 3·41 |
| 1·2 | 8·0 | 10·0 | 4·42 |
| Mean | | 9·3 | 4·232 |
| 3·3 | 40·0 | 2·85 | 1·30 |
| 3·4 | 37·3 | 4·75 | 2·16 |
| 3·5 | 31·0 | 4·36 | 1·99 |
| 4·2 | 38·0 | 5·15 | 2·32 |
| 4·6 | 43·0 | 4·9 | 2·24 |
| 5·85 | 53·0 | 3·88 | 1·33 |
| 6·0 | 53·3 | 4·0 | 1·81 |
| 7·4 | 69·0 | 3·62 | 1·54 |
| 7·7 | 82·0 | 4·99 | 2·26 |
| 8·2 | 82·0 | 4·53 | 2·07 |
| Mean | | 4·22 | 1·91 |

The relation we use is

$$\beta^3 K_1 = \Phi b^3 / M_c^{3/2} q \tag{2}$$

in which $\Phi$ is a constant ($2{\cdot}1 \times 10^{21}$), $q$ is the dispersion factor (1·95 for DNA), and $M_c$ and $b$ are the molecular weight and length of a segment respectively. Then we have from

$$\beta^3 K_1 = K M^{\alpha - 1/2} \tag{3}$$

(in which $\alpha$ is 1 for DNA [12]) the $K$ appearing in

$$[\eta] = K M \tag{4}$$

Table 1 (column 4) gives the value of $K$ found for various $M$, and so for various segments. All the values should be the same, because $K$ is a constant. But Table 2 shows that up to an $M$ of about $10^6$ the $K$ vary about a mean of $9 \times 10^{-6}$ (this result corresponds to the extended *B* form [12]); at higher $M$ the $K$ range on either side of $4{\cdot}22 \times 10^{-6}$, which is, as we have shown [13], the value for the compact form of DNA found in combination with protein. Therefore the most probable form for DNA of molecular weight over $10^6$ is the form found in DNP (the compact form). We ought to find that the DNA exists in the same form (or in a more compact one) when $M < 10^6$, because conditions then favour crystallization; but the value $9 \times 10^{-6}$ shows that

the DNA exists in the extended form. This contradiction is eliminated if we suppose that the *A* form exists when $M < 10^6$. Then, in our calculations of *b* and *K*, the vertical distance between nucleotides in DNA becomes 2·55 Å instead of 3·4 Å. Then (Table 3, column 5) the values of *K* range about $4{\cdot}23 \times 10^6$, which agrees very well with the value $4{\cdot}22 \times 10^6$ found for $M > 10^6$. The value $K = 1{\cdot}91 \times 10^6$ is meaningless, because it does not correspond to experiment.

TABLE 2. RELATION OF *K* TO *M* FOR DNA IN SOLUTION

| Published results [20] | | Calculated |
|---|---|---|
| *M* ($\times 10^{-6}$) | $[\eta]$ (100 cm³/g) | *K* ($\times 10^6$) |
| 0·3 | 1·77 | 5·90 |
| 0·45 | 3·12 | 6·95 |
| 0·7 | 5·35 | 7·65 |
| 3·4 | 37·3 | 10·95 |
| 7·4 | 69·3 | 9·33 |

VOL. 5 1960

Now in these calculations we have used the actual dimensions of DNA, so we conclude that DNA with $M < 10^6$ most probably exists in the compact form (the *A* form), and that DNA with $M > 10^6$ still has the compact form as its most probable form, but that here a certain amount of steric hindrance (caused perhaps by the solvent) is present. It may be that the *A* form is the preferred one when the space is filled by rigid rod-like segments and the proportion of solvent is not too large.

TABLE 3. SITE OF ACTION OF VARIOUS AGENTS THAT CAUSE DNA TO TAKE UP THE COMPACT CONFIGURATION

| Agent | H bond rupture | Suppression of phosphate dissociation | Rupture of phosphate ester bonds | Complexing (on phosphate groups) | Loss of structure in or removal of bound water | Loss of asymmetry during contraction (per cent) |
|---|---|---|---|---|---|---|
| Heat | + | — | — | — | + | 30 |
| Radiation | + | — | + | — | + | 30 |
| pH | + | + | — | — | + | 30 |
| Radiomimetic compounds | + | — | + | — | + | 30 |
| Protein | — | — | — | + | + | 30 |
| Choline chloride | — | — | — | + | + | 30 |
| Other cations | — | — | — | + | + | 30 |
| Reduction in relative humidity | — | — | — | — | + | 30 |

Our calculated value for *K* is approximately $4{\cdot}5 \times 10^6$, which agrees roughly with Perston and Peacock's [12] relation, $[\eta] = 4{\cdot}26 \times 10^{-6}\ M$ for fragments produced from DNA by ionizing radiation. This relation again points to the compact form as the preferred one.

We would expect to find that about 50 per cent of the molecules adopt the compact form when $M$ is of the order of $10^6$, because the molecules vary in $M$ and can adopt either form. The value of $K$ to be expected here is $0{\cdot}5\ (4{\cdot}5+9)\times10^{-6} = 6{\cdot}75\times10^{-6}$. Now Thomas [19] has found that DNAase splits DNA into fragments of about this molecular weight, and that $K$ is then $7\times10^{-6}$, which agrees well with our result. Lower values of $M$ naturally give $K$ closer to $4{\cdot}22\times10^{-6}$; $K$ increases with $M$ because the relative proportion of $A$ form decreases, a limit of $9\times10^{-6}$ being reached at $M > 10^6$ when the DNA is all in the $B$ form. Results [20] for $[\eta]$ as a function of $M$, which derive from light-scattering measurements on a series of homologous specimens of DNA (Table 2), confirm that $K$ increases up to $M \simeq 10^6$, but thereafter stays constant within the experimental error. We might well extend [12] the list of results that show that the $K$ values centre on $9\times10^{-6}$ for $M > 10^6$.

DNA exists in solution in the compact form if $M \leqslant 10^6$, and in the extended (metastable) form for $M > 10^6$, because crystallization is hindered. But external agents can cause the DNA to lose its metastable configuration; the compact, most stable form is then produced.

It is somewhat difficult to assess the degree of crystallinity of DNA in solution, but it is quite easy to establish the configuration adopted by the DNA. If the various agents cause the DNA to contract reversibly, and if the final form is the compact one, that form must be as regards those agents, more stable, than the initial one was. The same is true for DNA molecules individually; those with $M < 10^6$ must be configurationally more stable than those with $M > 10^6$.

These deductions are confirmed by tests on the reactions of proteins with DNA samples from various sources, and by stepwise deproteination tests on DNP [1–4]. Here the DNP contained about 20 per cent protein because the DNA was in the the 30 per cent contracted form. Further protein caused no further contraction. Therefore the compact form of DNA in relation to protein is more stable than the initial form is. The same effect is found when natural DNP is gradually deproteinated.

If we assume that the primary changes in the DNA are caused by the metastability of the extended form, we may further suppose that small doses of ionizing radiation can be responsible for making DNA contract from its extended state; therefore the compact form should show smaller changes in its molecular parameters than those shown by the extended form.

The $B$ form is damaged in two stages. First we get the reversible $B$ to $A$ transition, and subsequently we get irreversible denaturation of the $A$ form. Mild treatments produce only the first stage; if the treatment is not vigorous enough to cause degradation (the $A$ form is the more probable one under these conditions), the DNA appears more stable than it does in its $B$ form. This effect presumably is responsible for the protective behaviour of DNA. The large amount of structural water lost in response to the action of any agent may of itself precipitate contraction; the result of the contraction is that the DNA presents a target some ten times smaller than it did in the initial $B$ form. Figure 3 probably represents the response of DNA to such an agent.

The following test demonstrates that the compact form of DNA is the more stable one. First, by some means, we cause the DNA to contract; then we judge the relative stability of the two forms from the viscosity-change curves [21]. We made the DNA contract by 30 per cent by reducing the pH to 2·7–2·8; the transition to the compact form occurs very sharply in this pH range, because the DNA still exists entirely in the extended form at pH 2·9. Doses of 1500–2500 r reduce the viscosity of the extended form by 20 per cent, but the compact form shows no change at all, although the low pH favours the production of free radicals. The after-effects found with small doses are caused by subsequent contraction in the DNA; the compact form shows no after-effects.

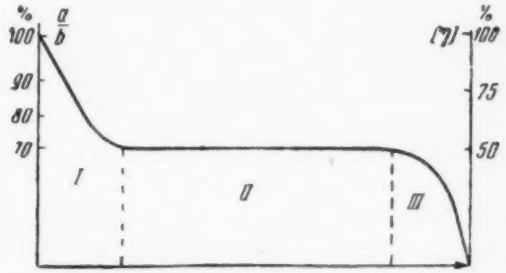


FIG. 3. Stability of DNA to agents causing changes in configuration. $a/b$ is the relative change in asymmetry, or the relative change in $[\eta]$; I is the region of the $A \rightleftarrows B$ transition, II is the region in which the compact $A$ form is stable, and in III dehydration occurs.

The effects at $M < 10^6$ are also important, because here the molecules should be in the $A$ form, or at any rate should be in a compact form. Experiments [22] show that doses of 2500–10,000 r cause a 30–50 per cent fall in viscosity if $M > 10^6$, whereas if $M < 10^6$ a sample treated with ultrasonics ($10^4$ c/s) shows no fall in viscosity and no after-effects; the same lack of effect is found in DNP, in which the DNA is present in the compact form [23]. Therefore in our terminology [1] the tertiary configuration of DNA found in DNP is configurationally more stable than the tertiary form of the initial (extended) DNA.

The compact form is often called denatured, but this is not correct, at least in our view, because the compact form is merely an intermediate stage when DNA really is denatured. It is wrong to say that DNA is denatured by being bound to protein, although the DNA is present in the DNP in compact form. Henstell, Freedman and Cooper [24] concluded that the physicochemical properties of DNP that has been heated are those often assigned to normal DNA; that is, the DNA is transformed to its native state from its denatured one when the DNP is denatured. This is, to say the least, unconvincing. We would rather say that DNA exists in two forms.

The results on the $C_1$ and $C_2$ forms show that the $C_1$ form obeys [12] our formula $[\eta] = 9 \times 10^{-6}\, M$, whereas for $C_2$, $[\eta] = 5{\cdot}5 \times 10^{-6}\, M$ [13]; that is, the second form is the compact one. The two forms also differ in molecular extinction coefficient at

2590 Å; $C_1$ gives values of 7400–7500, whereas $C_2$ gives 6600–6900. The lower values correspond to samples nearer to the native state [25]. Therefore $C_2$ has the compact configuration, and resembles the native substance more closely; the compact form cannot be a denatured one, at least.

We now examine why different agents produce the same compact form. Heat and low pH break hydrogen bonds that hold two polynucleotide chains together [26–28]; ionizing radiations and radiomimetic agents break these bonds also, and break the chains as well [29, 30]. The main result of combination between DNA and protein is that phosphate groups in the DNA are neutralized [1–4, 31]. None of these agents acts specifically; for example, the pH change must be such that about 75 per cent of the hydrogen bonds are broken, whereas far fewer such bonds must be broken to produce the same effect if the agent is urea or ionizing radiation. A fixed amount of protein (no matter what its amino-acid composition) is always required to cause DNA to contract, so the effect is not dependent on the number of phosphate groups neutralized. What is the cause of the relative stability of compact DNA to ionizing radiation at a pH such that most of the hydrogen bonds are broken? The extended form is not so stable even when most of its hydrogen bonds are intact. Why is DNA with $M < 10^6$ more stable to pH change and to radiation than is macromolecular DNA? The explanation can be given only in terms of some general process responsible for the contraction, because each agent produces not only contraction but also its own characteristic effects. Table 3 shows us that this general process is dehydration and loss of structure in the water bound to the molecule. The DNA crystallizes as a result of this loss of water; the *A* form (30 per cent contracted) results.

Jacobson [32–34] considers that many agents are capable of breaking the bonds between the DNA and the bound water, and thus of precipitating contraction (which results in falls in the dielectric constant, in the osmotic pressure, and in the viscosity). The changes are fairly slow because time is needed to alter the structure of the bound water. The DNA molecule binds the water because it fits ideally into the quasi-crystalline structure of water [32]. Here we refer to the bound water (water of hydration), which is held by hydrogen bonds to the DNA; the shell of ions, and this bound water, are probably responsible for the configuration taken up by DNA in solution [35]. Results on the dielectric parameters of solutions of DNA [34] are of special interest here; it is found that the DNA molecule can produce structure in the water out to 1000 Å from its surface, i.e. out to about a quarter of its root-mean-square radius or to about a twelfth of its length. Other results [36, 37] point to the fact that the molecule can bind a great deal of water.

Any agent (not too severe in its effects, of course) that disturbs this ordered layer of water may cause the metastable state to go over to one of higher entropy. Subsequently, if no crosslinks form within the molecule, the reverse process (restoration of the *B* form) should occur; in fact the extended form is regenerated when the disturbing agent is withdrawn. The loss of water can be detected when DNA combines with protein. Combinations usually have negative values of $\Delta S$, but Klotz [38] has shown that proteins give positive values of $\Delta S$ when they combine with anions; $\Delta S$ increases

VOL
5
196

with the size of the anion. Klotz explains this effect as caused by the loss of water consequent on the combination with the ions; that is, the combination results in more molecular units instead of fewer, and so the entropy of the product as whole increases. It is reasonable to suppose that DNA gives a similar effect, because the protein is strongly hydrated, especially near the cationic regions surrounding the nitrogen atoms, and those atoms take part directly in the combination. The most strongly hydrated parts of sodium DNA are the Na ions, each of which binds six of the eight $H_2O$ molecules bound per nucleotide to the DNA [39]. Much water must be lost as a result of the combination, because the most strongly hydrated parts play the major role in the combination.

The following facts confirm this argument. The water bound to 100 parts by weight of DNA or DNP is 52 for DNP and 95 for the *B* form of DNA [40], whereas the *A* form binds (at most) eight $H_2O$ molecules per nucleotide, i.e. about 43 parts by weight of water to 100 parts of DNA. Some of the water is bound to the protein, and the proportions of water in DNP and in *A*-type DNA are fairly similar; this again shows that the DNA in DNP is in the *A* form, and that the protein is in the $\alpha$ form. It also explains the 30% contraction of DNP relative to DNA.

Recent X-ray studies on DNA [7, 41] also indicate that the bound water plays a part, although here the Li salt was used instead of the Na salt of DNA. The configuration of the DNA changed only very slightly relative to the *B* form when the humidity was reduced to 44 per cent (the *C* form). Only Li ions prevent the transition to the *A* form; Na, K, and Rb do not. This effect can be explained in terms of the place occupied by Li in Hoffmeister's lyotropic series. Lithium has the smallest ionic mobility and the smallest atom, but its ions are the largest; that is, the field near a Li ion is stronger than that near any other singly-charged ion, H excepted. The result is that the strong field causes very marked hydration; by this we mean not a stoichiometric compound with water but a shell of oriented water molecules round each ion. The Li ions retain the water as the humidity is reduced, and so prevent the transition from the *B* form to the *A* form. This again shows that water plays a large part in the structure of DNA.

It can be shown directly that DNA fibres contract when they are dehydrated; a DNA fibre made in alcohol by the method one of us has described [42] always shows a contraction of 20–30 per cent. This shows again that the main process responsible for contraction is the loss of water.

Now DNA contracts in the same way, no matter whether the agent be protein or any other agent; again DNP [1–4, 43, 44] and compact DNA [28, 45] have molecular rigidities much lower than extended DNA does. This is a suitable point to deal briefly with the stability of the extended form; we consider that it is much less stable than many believe it to be. For example, it is stated that the activation energy for configuration changes is 30–100 kcal/mole, with the latter figure assigned to the initial form, on the assumption that the extended form is the more stable one [28, 46]. This is true if we assign the activation energy to rupture of hydrogen bonds between spirals, as some do [28, 46]. We consider that the main hydrogen bonds to break are those

at points at which there are breaks in the phosphate diester chains [1, 47]. Here the lower activation energy applies to fewer bonds (at those points only), but they occur in a less stable (but more intact) form of DNA. In the first case we need to break only 10 hydrogen bonds, whereas the second value corresponds to breaking about 30 hydrogen bonds, which may be present on account of damage done during the extraction of the DNA.

Finally we deal with some biological effects related perhaps to the two forms of DNA. The DNA from a phage may enter the cell as a result of considerable change in configuration in the DNA. The phage attaches itself to the cell; deproteination occurs, and the DNA straightens out (expands 30 per cent) and becomes metastable. The straight chain penetrates the cell; this penetrating form occurs not only when DNP is deproteinated, but also when DNA passes from the $C_2$ to the $C_1$ form (or from the *A* to the *B* form), especially since the $C_2$ form is characteristic of most types of bacterial DNA. The effect is vital to the transforming power of DNA, which is lost [48, 49] when the molecular weight exceeds about $10^6$. But DNA exists only in the compact form at lower molecular weights. Under these conditions the DNA cannot penetrate; it is, as it were, fixed in the compact form, whereas some penetrating power is needed to allow the DNA to enter the cell.

The compact form of DNA is more stable within the cell. Some of the effects caused by ionizing radiation at various stages in the mitotic cycle may be related to the difference between the sensitivities of the two forms of DNA. If the DNA is in the extended form, the radiation may cause an atypical nucleoprotein to form or may cause [40] the extended DNA to combine with a protein having the $\beta$ configuration (the compact form corresponds sterically to the $\alpha$ configuration [12, 13]). The atypical nucleoprotein may result in abnormal activities in the protein joined to the DNA, and may lead to inversion [1] and singular (DNA $\rightarrow$ DNP transition) points, and to other consequences well known from experiments with irradiated cells. Conversely. no atypical processes should occur if the normal DNP (containing DNA in the compact form) is produced; this is especially true for those structures whose main component is DNP (e.g. the chromosomes). In some cases ionizing radiation has relatively little effect on actively dividing cells [50]; i.e. one of the functions of the protein is to stabilize the DNA by producing the compact form. The above speculations require experimental confirmation; the inversion points found *in vitro* for DNP should be compared *in vivo* with the various transformation stages of DNA and of DNP, the main component of chromosomes.

The halving of the viscosity of DNA as a result of treatment with entirely different agents has thrown light on the changes caused by ionizing radiation, on the alterations in the structure of the bound water, and on the transition from the *B* form to the *A* form; it has also revealed the mechanism of the after-effects found in DNA treated with quite small doses of penetrating radiation.

The distance between corresponding planes of the nitrogen bases and sugars is reduced by 30 per cent when the *B* form goes over to the *A* form; this contraction must accentuate the interactions of the various parts of the molecule. The 20 per cent

protein in DNP [1] is a sort of safety device, which must be removed before damaging agents can act on the DNA. These arguments do not clear up the mechanisms or consequences of the molecular transitions in DNA, but our hypotheses are useful working ones, even though they relate to only one of the possible configurations. More detailed work on the exact form of the DNA molecule in solution will enable us to describe exactly the two forms of DNA, which can be transformed one into the other, and which differ in that one has twice the viscosity of the other.

*Translated by* J. E. S. BRADLEY

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* There is a translation into English of this work.

# THE ROLE OF MATRIX STRUCTURES IN REPLICATION*

A. G. PASYNSKII

A. N. Bakh Institute of Biochemistry, U.S.S.R. Academy of Sciences, Moscow

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THE remarkable accuracy of the replication of specific structures in biosynthetic processes, most evident in phenomena associated with inheritance, is usually explained in terms of matrix structures (DNA, RNA, nucleoproteins, etc.), which form templates for the formation in the cell of new amino-acid or protein macromolecules of identical or complimentary specific structure. Owing to the lack of suitable factual data, these concepts have been developed on the basis of qualitative correlations, whose nature is as yet unclear; some data have appeared in recent years, however, which can be utilized in a preliminary quantitative evaluation of this question.

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VOL
5
196

Study of the sequential arrangement of amino-acids in protein molecules, which to a large extent is concerned with the problem of their specificity, has demonstrated the very high accuracy of replication of this sequence. Sanger [1] has stated that no peptides were found following hydrolysis of insulin which could not be fitted into A and B chains of 21 and 30 residues respectively; at the same time it has been found that the structure of the *B* chain from different species of animals was identical, whereas the A chain differed in one amino-acid per intramolecular disulphide bond (e.g. replacement of serine by glycine in position 9 in the case of bovine and sheep insulin). Thus a difference of one amino-acid in 20 residues (i.e. about 5 per cent) can be determined experimentally. True, these figures refer to the total mass of the molecules studied and they are not indicative of any deviations in the structure of a small proportion of molecules which might possibly occur during biosynthesis. In view of the thoroughness with which the determination of the sequence of the amino-acid residues has been carried out, however, we can take a value of 5 per cent, or even 1 per cent, as the upper limit of biosynthetic "error" for the chain as a whole. It is possible that this value is even lower, but on the other hand the error must have a finite value, since it would be impossible to explain a zero value by any physiological mechanism subject to statistical variation, ignoring the unlikelihood of such a hypothesis from a biological standpoint.

VOL. 5 1960

An error of 1 per cent in the biosynthesis of a protein molecule containing 100–300 residues per chain, for example, is very difficult to explain since it represents an error of 0·01–0·003 per cent only per single link of the chain*. The question arises as to the nature of the physico-chemical mechanism which ensures an accuracy of selection of amino-acids of this order.

Pauling [3] was the first to attempt to calculate the error of selection for a matrix structure in the case of a choice between glycine and alanine, for example. He suggested that the part of the matrix complimentary to alanine must have a receptor for a methyl group sufficiently small to reject all amino-acids other than alanine or glycine; alanine is accepted in preference to glycine on account of the stronger Van der Waals' attraction between the matrix and a methyl group than between it and a hydrogen atom. By calculating the difference in energies responsible for the preferential choice of alanine, Pauling was able to conclude that the probability of an error involving the replacement of alanine by glycine would be about 5 per cent, although in his opinion this figure could be taken as 1 per cent. The figure calculated by Pauling relates to the individual links of the chain, and not to the chain as a whole, therefore this figure is 2–3 orders of magnitude greater than the figure which is in need of explanation. Since steric and energetic factors are included in Pauling's calculation (i.e. all aspects of matrix structure), and since the nature of the forces and bonds in protein and nucleic acid macromolecules are in general the same, this conclusion applies to any matrix structure found in the cell.

* This value can be compared with the probability of occurrence of a single genetic mutation (approximately $2 \cdot 10^{-5}$–$10^{-6}$ for man [2]) and biosynthetic errors may be of substantial importance in the causation of genetic mutations.

The inability to explain the real value of the biosynthetic error (less than 0·01–0·003 per cent per link) by this means evidently arises from the fact that Pauling considered the choice of amino-acids by a matrix structure as an equilibrium process. In this case the following equations can be applied to the adsorption of activated amino-acids $A$ and $B$ on centres A of a matrix structure:

$$\begin{aligned} \Delta F_{AA} &= RT \ln K_A, \\ \Delta F_{BA} &= RT \ln K_B, \end{aligned} \tag{1}$$

where the equilibrium constants $K_A$ and $K_B$ are equal to the ratio of the amount of amino-acid $A$ and $B$ bound to centre A to their concentrations in the solution.

$$K = \frac{C_{A\ \text{bound}}}{C_{A\ \text{sol}}} \text{ and } K_B = \frac{C_{B\ \text{bound}}}{C_{B\ \text{sol}}}$$

Where there are equimolar concentrations of the amino-acids in solution ($C_{A\ \text{sol}} \simeq C_{B\ \text{sol}}$) it follows from equation (1) that:

$$\Delta F_{AA} = \Delta F_{BA} = \sigma \Delta F_A = RT \ln \frac{K_A}{K_B}. \tag{2}$$

It has been established experimentally that the energies of combination $\sigma \Delta F_A$ of such related, but at the same time dissimilar, compounds as butyric and caprylic acids, or octyl sulphate and dodecyl sulphate, with proteins are about 1·5–2·0 kcal/mole [4] (unfortunately there are no figures available for amino-acids and nucleotides), therefore differences of more than 2–3 kcal/mole in the free energy of combination of two activated amino-acids would not be expected.

Substituting a value of 3 kcal/mole for $\sigma \Delta F_A$ in equation (2) we find that $3 = 0{\cdot}6 \times 2{\cdot}3 \ln K_A/K_B$ and hence $K_A/K_B \simeq 100$, which corresponds to the lowest error value suggested by Pauling — about 1 per cent — whereas the 5 per cent error value calculated by Pauling corresponds exactly to a figure of 2·0 kcal/mole for $\sigma \Delta F_A$ (Pauling does not indicate the method of calculation in his paper). It can readily be seen that it is impossible to obtain the required biosynthetic error of 0·01–0·001 per cent by this means, since it would be necessary to assume improbably large differences of the order of 6–8 kcal/mole in $\sigma \Delta F$, the free energies of combination, of two, sometimes closely related, activated amino-acids. Various other hypotheses likening the adsorption of amino-acids on specific sectors of a matrix structure to a process of crystallization are unsatisfactory for the same reason [5]. Hypotheses of a similar nature have been suggested to explain the incorporation of bases into a DNA chain of the Watson–Crick pattern, or the selection of amino acids [6] or their activated combination with nucleotides [7] by means of intermolecular forces of attraction (hydrogen bonds and Van der Waals' forces), taking into account steric limiting factors. All these hypotheses assume the existence of equilibrium states, and they are attended by the same difficulties as Pauling's calculation. The position is substantially unaltered even if phos-

phoric ester bonds are suggested as the means of binding the activated amino-acids to the nucleic acid matrix [8, 9], since in view of the known energies of these bonds, values of 6–8 kcal/mole for $\sigma\Delta F$ are exceptional and in fact rarely exceed 2–3 kcal/mole.

In our opinion the problem cannot be resolved without consideration of the kinetics of the process, and for this purpose it is necessary to construct a sufficiently plausible hypothesis whereby alignment of amino-acids or nucleotides during condensation on a matrix structure need not be regarded as a thermodynamic equilibrium, but rather as a kinetic process with an activation barrier $E$. The idea that the biosynthesis of protein should be regarded as a kinetic problem was first advanced by us in 1951 [10], although at this time much of the appropriate data, including Sanger's work, was not available. It is now possible to derive formulae for the rate of orientative adsorption of activated amino-acids $A$ and $B$ on centre A of a matrix structure as follows:

$$V_{AA} = P_{AA} \exp^{(-E_{AA}/RT)},$$
$$V_{BA} = P_{BA} \exp^{(-E_{BA}/RT)}, \tag{3}$$

hence

$$\frac{V_{AA}}{V_{BA}} = \frac{P_{AA}}{P_{BA}} \exp^{(-\Delta E_A/RT)}, \tag{4}$$

where the ratio of the steric factors $P_{AA}/P_{BA}$, according to the theory of absolute rates of reactions, is dependent on the entropy factor of activation $\Delta S^*$:

$$\frac{P_{AA}}{P_{BA}} = \exp\left(\frac{\sigma\Delta S^*}{R}\right). \tag{5}$$

An error of 0·01–0·001 per cent corresponds to a value of $V_{AA}/V_{BA} = 10^4$–$10^5$, and from equation (4) to a value for $\Delta E = 6$–$8$ kcal/mole (where $P_{AA}/P_{BA} \simeq$ ), and activation barrier differences of this sort are quite probable, since the height of the activation barrier can be considerably greater than this value (e.g. $E > 21$ kcal/mole for hydrolysis of a peptide bond [12]), thus leaving scope for interpretation of even smaller biosynthetic errors. On the other hand, according to equations (4) and (5), the steric factors can be responsible for large differences in $V_{AA}/V_{BA}$, e.g. where $\sigma\Delta S^*_{11} = 15$–$18$ entropy units $P_{AA}/P_{BA} \sim 10^4$; a difference of this sort in the value of $\Delta S^*_{11}$ is also quite feasible since during denaturation for instance there can be 12–28 entropy units difference in the value of $\Delta S^*$ per broken hydrogen bond [11]. A biosynthetic error of 0·01–0·001 per cent can also be explained by a suitable combination of the differences of $\Delta E (\sim \Delta H^*)$ and $\Delta S^*$ — the energy and entropy of activation of the process of stereometric arrangement on the matrix structure; since during biosynthesis the real values of these terms are unknown, however, the approximate estimates given above are of use mainly in indicating the various modes of action of the matrix structure which can be excluded. In particular the known accuracy of biosynthesis cannot be explained by a thermodynamic equilibrium and it may be

supposed, contrary to current hypotheses, that the matrix structure does not function simply as a selective adsorbent or crystallization template.

New experimental approaches can be designed and certain aspects of replication explained by a kinetic treatment of the role of the matrix structure. The phenomenon of the multiplication of phage in a bacterial cell is of especial interest, since it involves an alteration of biosynthetic processes with the formation of new types of protein and DNA. It has been found that the biosynthesis of phage proceeds at the expense of the amino-acids of the medium as well as those of the host cell, and that the DNA of the introduced phage breaks up into a number of relatively large fragments, each of which becomes the focal point for the formation of a new phage particle (similar facts have been established in the case of a number of viruses). In a series of classical papers by Northrop and his colleagues [13; 14], it has been shown that the rate of multiplication of bacteriophage $(P/P_0)$ may be a hundred times greater than that of the host bacterium $(B/B_0)$, such that $(P/P_0) = (B/B_0)^n$, where $n \gg 1$. The normal enzymes of the host bacterium participate in the formation of phage; this occurs mainly during the growth phase and it is not observed in dead cells.

VOL
5
196

In the case of cancer cells there is an observable increase in the rate of biosynthesis, an increased rate of formation of atypical cells with certain common morphological characteristics being a general feature of all forms of cancer [15].

In general, distortion of a normal biosynthetic process leading to replication of an anomalous or foreign matrix structure is always the result of the predominance or equivalence of the rates of biosynthetic pathways.

The biosynthesis of protein is a multi-stage process; activation of the carboxyl groups of the amino-acids, their combination with soluble cytoplasmic RNA or nucleotides, transfer to the matrix, attachment of the amino-acids to the matrix by peptide bonds, separation from the matrix and the formation of a three-dimensional structure. The stereometric arrangement of activated amino-acids on the matrix structure must be regarded as the stage which limits the over-all rate of biosynthesis, since the preceding stages of activation of the amino-acids provide sufficient energy for the process of condensation to proceed spontaneously. Further, it may be supposed that the excess of free energy (approximately 2–3 kcal/mole) involved in condensation of the activated amino-acids is exactly balanced by the energy expended during separation from the matrix immediately after the reaction, the result being that only the ends of the chain remain attached to the matrix; consequently only completed protein molecules and low molecular weight substances are normally observed in the cell. Spontaneous steric deformation or folding of the chain, depending on the chemical composition of the links of the chain and the sequential arrangement of their components, may occur simultaneously with separation from the matrix. It is difficult to accept that an introduced DNA macromolecule can affect the activation of amino-acids (enzymatic reactions with ATP, soluble RNA, etc.), which may occur at a considerable distance from it, or the diffusion of amino-acids to the matrix. Consequently the increased rate of biosynthesis mentioned above, which follows the introduction of phage or viral DNA is probably closely connected with the stage of orientative

attachment to the matrix structure*. This conclusion also provides an explanation for the fact that strongly depolymerized DNA does not affect biosynthetic processes as much as highly polymerized DNA, although the grouping of loci is unaltered over considerable sections. In this case the need for diffusion of biosynthetic products from one section of DNA to another retards the process to such an extent that it cannot compete with the normal rate of biosynthesis on the intact original structure where no such diffusion is required. Undoubtedly the appearance of matrix structures in coacervates or in other open systems during the course of evolution was a new development of great significance, not only in providing an inexhaustible source of specific structure but also because it obviated the necessity for transportation by diffusion and thus produced an over-all acceleration of biosynthetic processes in comparison with the state of affairs in a homogeneous solution of enzymes for instance.

*Translated by* C. PRINGLE

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960

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* According to Loftfield [16], the concentration of activated amino-acids in a cell is usually many times greater than the concentration necessary for the rate of biosynthesis of protein observed, from which it follows that differences in reaction rates during the enzymatic activation of amino-acids are of subsidiary importance, although naturally all stages in biosynthesis are equally neccessary and one cannot oppose another; basically they are all, including the "matrix", kinetic phenomena.

# OXIDATION PROCESSES OF BIOLOGICALLY-ACTIVE SUBSTANCES IN A FIELD OF ULTRASONIC WAVES*

I. Ye. EL'PINER and A. V. SOKOL'SKAIA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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A NUMBER of investigations have shown that in a field of ultrasonic waves biologically-active substances undergo various chemical transformations: reduction, oxidation, polymerization and depolymerization [1, 2]. In the present communication we shall concern ourselves only with problems related to ultrasonic oxidation of some substances of biological importance.

According to the available data, there occurs in the cavitation bulb, formed as a result of ultrasonic irradiation, not only ionization of the water molecules but also ionization or activation of any gases present. This explains the fact that in irradiated water ammonia is formed in the presence of $N_2$ and $H_2$ and hydrocyanic acid and formaldehyde in the presence of $N_2$, CO and $H_2$ [3].

The oxidation processes are associated not only with the appearance in the reaction mixture of OH radicals, but with activation of oxygen molecules. Activated oxygen either enters the reaction directly or by forming the $HO_2$ radical ($H+O_2 \rightarrow HO_2$) which somewhat intensifies the oxidative processes in the ultrasonic field. However, as will be shown, intensification of the oxidative processes in irradiated water may occur in a number of cases in the absence of oxygen. We were able to observe this on replacing oxygen by certain inert gases. Moreover, it was found that the inert gases vary among themselves in the degree and character of their influence on the course of the oxidative processes in the ultrasonic field. Argon and helium differ sharply in this respect and form the subject of our study.

It is important to point out that attempts have been made to explain the different behaviour of these gases by their different ionization potentials or degree of solubility in water [4]. However, there are grounds for believing that the inert gases activated or ionized in the cavitation bulb obviously differ in their action on the OH radicals produced by ionization of the water molecules in the bulb, and that they exert a specific influence on the chemical reactivity of these radicals.

As far back as 1936 Neimin and Terenin [5] reported spectral data showing that argon and helium act differently in respect to hydroxyl radicals produced by ultra-violet rays. It is possible that helium in contradistinction to argon inactivates the free radicals and thereby depresses the oxidative processes in insonated water. Argon, even in

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the absence of oxygen, does not prevent and may even promote the oxidizing effect of ultrasonic waves.

Thus, by studying the chemical conversions of biologically-active substances in the presence of various gases, either active or inert, it is possible to judge whether the processes of ultrasonic oxidation are mediated by activated oxygen, by the OH radicals or by both these oxidants. This opens up new avenues in the study of elementary processes underlying the oxidation of biologically-active substances induced by various physical agents: ionizing radiations, ultra-violet rays and ultrasonic waves, etc.

## EXPERIMENTAL SECTION AND DISCUSSION OF RESULTS

The test substances were proteins, amino-acids, nucleic acids, carbohydrates and other compounds. The criterion on which the intensity of the chemical process was assessed was the amount of formaldehyde produced in the given conditions. We would recall that according to a number of investigators, aldehydes, and in particular, formaldehyde, are evolved when solutions of the substances enumerated are irradiated with ultrasonic waves [6, 7]. The presence of formaldehyde in solution was determined by its reaction with phenylhydrazine and an alkaline solution of pottassium ferricyanide.

In a number of cases the chemical conversions of the substances under study induced by ultra-sound were estimated by spectrophotometric investigations in the ultra-violet.

The active and inert gases were freed from oxygen in a specially-assembled column proposed by Kanotorovich and Rapoport [8]. The frequency of ultra-sound was 380 kc/s and the intensity 3–4 W/cm$^2$.

TABLE 1. INFLUENCE OF THE GASEOUS MEDIUM ON YIELD OF FORMALDEHYDE FORMED IN IRRADIATED AQUEOUS SOLUTIONS OF PROTEIN, AMINO-ACIDS AND OTHER ORGANIC SUBSTANCES

| Substance | Duration of irradiation (min) | Amount of formaldehyde in presence of various gases (μg/ml) | | |
|---|---|---|---|---|
| | | Air | Argon | Helium |
| Alanine | | 0·8 | 2·6 | 1·0 |
| $CH_3$—CH—COOH | 180 | 0·2 | 2·2 | 0·6 |
| $NH_2$ | | | | |
| Serine | | | 3·2 | 1·0 |
| $CH_2$—CH—COOH | 180 | 1·4 | 2·4 | 1·0 |
| OH $NH_2$ | | 1·6 | 3·0 | 1·0 |
| Aspartic acid | 180 | 1·0 | 3·0 | 0·6 |
| COOH—CH—$CH_2$—COOH | | 1·2 | 3·2 | 0·8 |
| $NH_2$ | | | | |
| Ketoglutaric acid | | 2·4 | 16·8 | 2·0 |
| HOOC—$CH_2$—$CH_2$—C—COOH | 120 | 2·2 | 15·2 | 3·4 |
| 0 | | 1·6 | 14·2 | — |
| Serum albumin | | 0·2 | 2·2 | 1·0 |
| | 60 | 0·2 | 3·2 | 1·2 |
| | | 0·4 | 3·0 | 1·0 |

As Table 1 shows, in the aqueous solutions of a number of amino-acids saturated with oxygen, formaldehyde was produced by the effect of ultrasonic waves. However, the largest amount of formaldehyde was observed when the solutions prior to irradiation were saturated with argon. Saturation with helium rarely led to an increase in the rate at which this substance split off the amino-acids.

Similar results were obtained in studying the rate of formation of formaldehyde in irradiated aqueous solutions of α-ketoglutaric acid. In this case the yield of formaldehyde in the presence of air was 1·8–1·6 μg/ml. The yield was, however, increased several times on introduction of argon into the solution instead of air (16·8–14·0 μg/ml), whereas helium was less active in this connexion.

The same pattern was also obtained with ovalbumin and serium albumin. In addition, the protein coagulation effect of ultrasonic waves was considerably enhanced. Even serum albumin, which usually requires prolonged irradiation (3–4 hr) before coagulation occurs, gave a protein precipitate within only 60 min when irradiated in the presence of argon. In the case of helium, we gained the impression that this gas somewhat depresses the process of protein coagulation in the ultrasonic field.

The effect of ultrasonic waves on methylene chloride, $CH_2Cl_2$, was to give a new organic compound which precipitated in the solid state and had the empirical formula $C_{10}H_7O_3Cl_2$. Infra-red spectrometry showed the substance incorporated C = O, C = C and CH radicals. It is important to note that the intensity of the synthesis described was also dependent on the nature of the gas used to saturate the medium before irradiation. As Fig. 1 shows, synthesis of $C_{10}H_7O_3Cl_2$ induced by ultrasonic

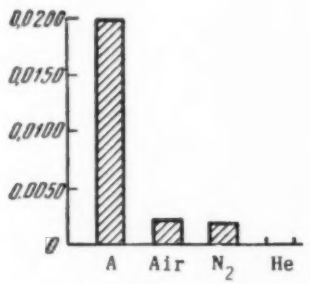


Fig. 1. Effect of ultra-sound on the mixture $H_2O+CH_2Cl_2$ when saturated with argon, air, nitrogen and helium. Original suspension diluted 30 times; irradiation time 5 min; ordinates, degree of turbidity.

waves proceeded slowly in a nitrogen medium, somewhat more rapidly in air and quite quickly in argon, whereas helium in contrast severely depressed this synthesis. If we bear in mind that the new synthetic product includes oxygen in its structure, then the source of the latter in the presence of argon must have been the ionization products of the water molecules (OH radicals) since during the preliminary saturation of the solution with argon, the oxygen was expelled from the test liquid (verified polarographically).

We have information indicating that not only helium but also argon depresses oxidative processes in certain cases, e.g. the ability of both inert gases to depress the reaction in which iodine is liberated from aqueous solution of potassium iodide (Table 2). In this reaction, consequently, the prime pre-requisite is the presence of oxygen in the solution.

TABLE 2. RATE OF LIBERATION OF IODINE FROM POTASSIUM IODIDE IN RELATION TO GAS PRESENT IN THE IRRADIATED SOLUTION

| Gas used to saturate 1% aqueous solution of KI | Amount of iodine formed in 1% of KI solution after irradiation for 20 min (μg/ml) |
|---|---|
| Air | 250 |
| ,, | 400 |
| Argon | 21 |
| ,, | 44 |
| ,, | 16 |
| Helium | 5 |
| ,, | 5 |
| ,, | 5 |

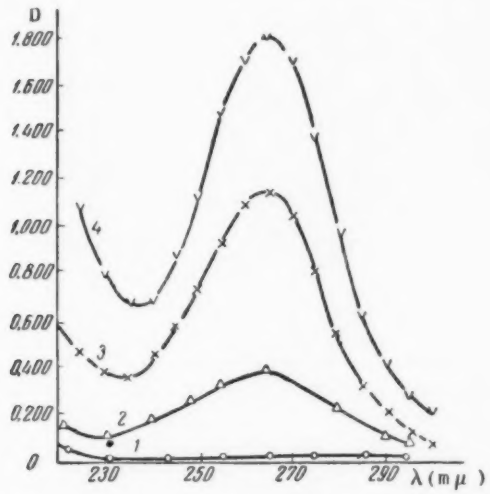


FIG. 2. Spectrophotometric curves of dependence of optical density of irradiated solutions of glucose on the nature of the gas present. (1) before irradiation; (2) 20 min irradiation in helium; (3) in oxygen; (4) in argon. Solvent, 0·1 N NaOH; concentration 6 mg per 10 ml.

We give another example indicating the predominant role of the OH radicals in the oxidation of organic substances. Recently we were able to demonstrate that carbohydrates (glucose, sucrose, raffinose) in alkaline medium undergo chemical conversions under the influence of ultrasonic waves leading to the appearance of

substances with maximum absorption in the ultra-violet region of the light spectrum. As Fig. 2 shows, there was a considerable increase in the absorption maximum for glucose when irradiated in the presence of argon.

Other facts also suggest that inert gases may influence the kinetics of chemical reactions induced by ultra-sound. Fig. 3 shows the spectrophotometric absorption curves for 0·2 mg adenine in 10 ml of 0·14 M NaCl irradiated in the presence of the various gases. As can be seen, spectrophotometrically there was almost complete disappearance of adenine after irradiation in the presence of $O_2$ for 60 min (the oxygen was obtained by decomposition of $KMnO_4$). A smaller amount of adenine was destroyed on irradiation in the presence of argon, while no chemical reaction took place with

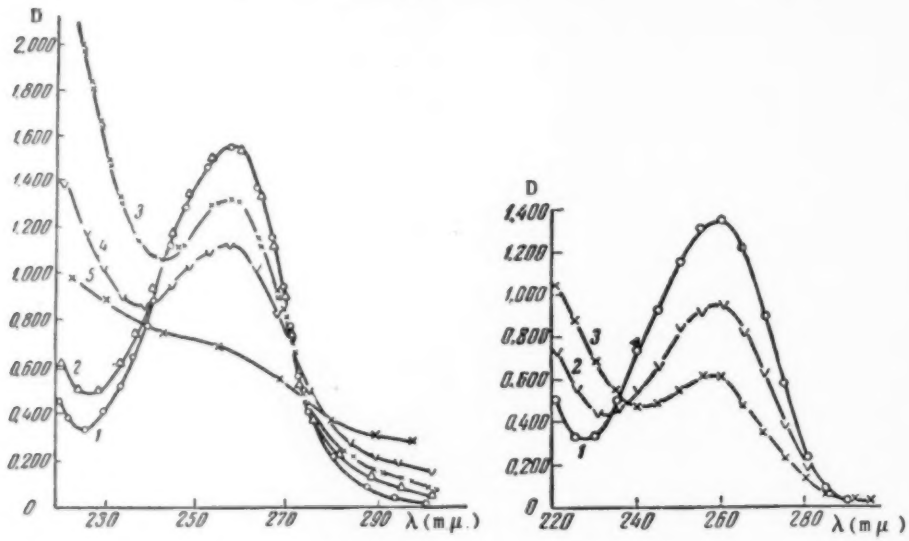


Fig. 3. Spectrophotometric curves of dependence of optical density of irradiated solutions of adenine on nature of gas present. Solvent, 0·14 M NaCl; concentration 0·2 mg per 10 ml: (1) before irradiation, (2) 60 min irradiation in helium, (3) in air, (4) in argon, (5) in oxygen.

Fig. 4. Spectrophotometric curves of dependence of optical density of irradiated solutions of uracil on nature of gas present. Solvent 0·14 M NaCl; concentration 0·2 mg per 10 ml: (1) before irradiation, (2) 60 min irradiation in argon, (3) in oxygen.

preliminary saturation of the irradiated solution with helium. This shows that oxidation of the purine base, in the case in question, is mediated chiefly by the oxygen present in the water, which is apparently activated in the cabitation bulb. It must be considered that the OH groupings, combinations of which may give a certain amount of oxygen, play a lesser role in this process. As Fig. 3 shows, the effect was less conspicuous in air than in oxygen since here apparently the competing action of other gases comes into play and, in particular, that of nitrogen.

We also obtained similar findings on irradiating uracil in the presence of these different gases, although the yield of the reaction was lower in this case (Fig. 4).

In the investigation of adenosine triphosphate a somewhat different picture was observed. We would first point out that the more complex the molecule incorporating the purine or pyrimidine bases, the more stable the molecule in the field of ultrasonic waves or, more accurately, the smaller its ability to interact with oxygen and the ionization products of water. The same observations have been made by Rysina and Libinzon [9] who investigated the influence of ionizing radiations on aqueous solutions of this type of substance. According to our findings, ATP undergoes a certain amount of destruction in the ultrasonic field if the irradiation is carried out in the presence of pure oxygen or argon (Fig. 5). This apparently implies that the oxidation of ATP is about the same both in the presence of $O_2$ and with the appearance of OH radicals in the presence of argon. However, the purine ring of ATP is more stable to ultrasound than is the purine ring of adenine.

VOL. 5 1960

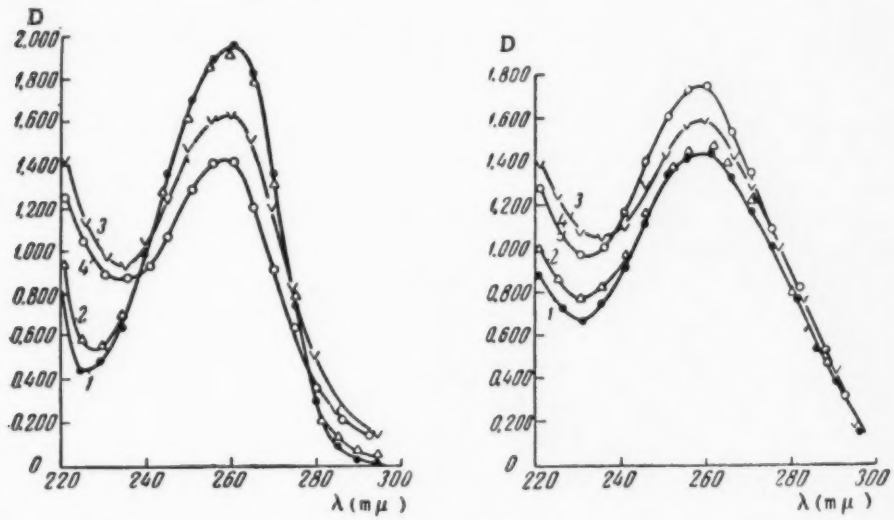


FIG. 5. Spectrophotometric curves of dependence of optical density of irradiated solutions of adenosine triphosphate on nature of gas present. Solvent, 0·14 M NaCl; concentration, 1 mg per 10 ml: (1) before insonation, (2) 60 min insonation in helium, (3) in argon, (4) in oxygen.

FIG. 6. Spectrophotometric curves of dependence of optical density of irradiated solutions of DNA on the nature of the gas present. Solvent, 0·14 M NaCl; concentration, 1 mg per 10 ml: (1) before irradiation, (2) 60 min irradiation in helium, (3) in argon, (4) in oxygen.

Finally, with even higher complexes of the molecules under study, for example, on irradiation of dexyribonucleic acid, the purine and purimidine bases (at the exposures employed by us) showed no interaction whatsoever with $O_2$. In the case of the OH radicals formed in the presence of argon, they produced in our irradiation conditions only slight destruction of these rings (Fig. 6). As this figure shows, there was an increase in the optical density of the solution over the entire ultra-violet spectrum in the presence of oxygen but the absorption maximum was unchanged. A certain displacement of

the absorption maximum was observed in investigating nucleic acid solutions irradiated in the presence of argon.

The results presented here are of great interest not merely because they indicate a possible means of regulating the course of chemical processes by artificial procedures and exerting an influence on the yield of the reactions but also by the fact that they open up new prospects in the interpretation of the mechanism of oxidation and other actions of physical factors possessing so-called indirect effects.

## SUMMARY

(1) The intensity of the oxidative reactions of amino-acids, glucose, purine and pyrimidine bases and proteins induced by ultrasonic waves is dependent on the nature of the active or inert gas present.

(2) The processes of oxidation of biologically-active substances occur in the field of ultrasonic waves not only in the presence of oxygen but also in its absence when replaced by argon (dependent on the structure of the test substance), whereas helium is inert in this respect.

(3) It is possible to judge by studying the oxidative effects of ultrasonic waves in the presence of these gases whether oxygen itself or the OH radical plays the dominant role in this process.

(4) It is postulated that the inert gases, argon and helium activated in the cavitation bulb have different effects on the chemical activity of the OH radicals — the ionization products of the water molecule — present here.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# A PHOTOELECTRIC STUDY OF THE KINETICS OF OXYHAEMOGLOBIN CLEAVAGE UNDER THE ACTION OF ALKALIS AND ACIDS*

M. S. LEVINSON and B. G. KOVROV

Institute of Physics, U.S.S.R. Academy of Sciences, Krasnoiarsk

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UNDER the action of dilute mineral acids or of alkalis, oxyhaemoglobin dissociates into a prosthetic group and a protein residue. It was originally shown by Krüger [1] that when alkali acts on haemoglobin, denaturation occurs at rates that differ appreciably according to the species from which the haemoglobin was obtained.

Vakulenko [2] also determined the stability of haemoglobin to alkali and to acetic acid. In his experiments the time required for the complete disappearance of the absorption band of oxyhaemoglobin in the spectrum was noted. Vakulenko found a difference in the stability of haemoglobin to alkali and acid in different species of animals, in man, and in some pathological states. In particular, he observed a marked fall in the stability of oxyhaemoglobin in typhus cases. The work of Vakulenko showed that for a constant concentration of alkali, or of acetic acid, the time required for the decomposition of oxyhaemoglobin from different animals and also from normal and pathological human subjects differed sharply. The difference in stability between species was considerably less for acetic-acid decomposition than for alkali decomposition. The alkali stability of individual haemoglobins was evidently not related to the acid stability; for example, the haemoglobin of the ox showed a greater stability to alkali than that of the swine, but the swine haemoglobin was more stable to acids.

The results of Bliumenfel'd and Krasovitska [3] confirm those of Vakulenko. Some divergencies between the two sets of results are evidently due to imperfections in Vakulenko's methods. Later many workers [4–10] studied the stability of haemoglobin to alkali in adults, in the foetus, and in human subjects with pathological conditions.

In oxyhaemoglobin decomposition studies, generally only the time required for complete decomposition is taken, and the nature and special features of the process have received little attention. There is a good deal of information to indicate that in different molecules of oxyhaemoglobin, even from the same animal, the bonds between the haems and the globin are dissimilar. Moreover, because of the possibility of "haem-haem" interaction, the rupture of one of the haems from the globin must affect the strength of the bonds of the other haems with with the globin [11]. All this

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should be reflected in the kinetics of oxyhaemoglobin decomposition. An example described in the literature of the weakening of the bonds between the haems and the globin in a pathological state also indicates the possibility of haemoglobin molecules differing in their stability.

Photoelectric measurements of the changes in the absorption spectrum enable the kinetics of the transition of oxyhaemoglobin ($HbO_2$) to haematin to be studied with sufficient accuracy, and the decomposition curve of oxyhaemoglobin can also be obtained.

The object of the present work was to attempt to find differences in the kinetics of oxyhaemoglobin decomposition in normal blood and in blood associated with various pathological states. Acetic acid and sodium hydroxide were used to promote the decomposition of haemoglobin. The two reagents were used in order that differences in the mechanism of decomposition by alkalis, and by acids might be elucidated.

The rate of oxyhaemoglobin decomposition was studied by recording the time course of the optical density at 576 mμ of a solution of $HbO_2$ subjected to the action of alkali or of acetic acid.

The following procedure was used. A 5 ml glass cuvette with double walls was placed at the exit slit of a UM-2 monochromator. Distilled water, maintained at a constant temperature by a TS-15 thermostat, was passed between the walls. The light source was fed from an accumulator. A section of the spectrum in the region of the maximum of the α-absorption band of oxyhaemoglobin ($\lambda = 576$ mμ) was isolated by means of the monochromator. The width of both exit and entrance slits was about 0·25 mm. A selenium photocell was placed directly behind the exit slit; this was connected to a mirror galvanometer (EPS-46) having a sensitivity of $1{\cdot}9 \times 10^{-9}$ A/mm/m, and a characteristic vibration period of 0·85 seconds. The blood sample (about 0·1 ml) was haemolysed with distilled water (about 2 ml) and centrifuged to remove stromata.

At the beginning of each experiment the constant-temperature cuvette was filled with distilled water. The galvanometer was set at 400 units ($I_o$) by adjusting the exit slit. A solution of oxyhaemoglobin was then added to the water until the deflection was reduced to 100 units; a constant concentration was achieved in this way. A volume of 2 ml of the resulting solution was left in the cuvette and 2 ml of the reagent for decomposing oxyhaemoglobin was added. After 2 to 3 sec, which was necessary for mixing of the solutions, the first galvanometer reading was taken and a stop-watch was started. All the remaining readings ($I_t$) were taken at intervals of one minute, or more frequently if the circumstances required it. The observations were continued to the end of the reaction, which was determined by the cessation of changes in the optical density (final reading of the galvanometer ($I_f$)).

The percentage of $HbO_2$ decomposed was expressed by the equation

$$C = \frac{D_i - D_t}{D_i - D_f} \times 100\% \qquad (1)$$

Since

$$D_i = \log \frac{I_o}{I_i}, \quad D_f = \log \frac{I_o}{I_f}, \quad D_t = \log \frac{I_o}{I_t},$$

equation (1) can be written in a form more convenient for calculations

$$C = \frac{\log \frac{I_t}{I_i}}{\log \frac{I_f}{I_i}} \times 100\% \qquad (2)$$

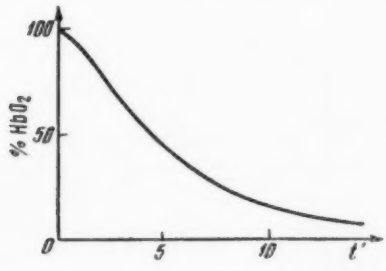
The concentrations of acid and alkali in the experiments described were selected to give a reaction time of about 15 min. This period was chosen partly because in this time the measurements would not be affected by the comparatively slow aggregation of haemin molecules. The method was used to study the decomposition of haemoglobin from healthy human subjects, from some subjects suffering from diseases, and also the decomposition of foetal and new-born haemoglobin.

The experiments with acetic acid at a concentration of 0·1 *N* showed that the differences in acid stability for haemoglobin from different subjects were not great. Blood from three male and three female subjects was examined. Table 1 gives the mean results of these experiments.

TABLE 1

| Time (min) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % $HbO_2$ remaining (100 — *C*) | 92·1 | 79·5 | 66·3 | 54·9 | 44·4 | 36·3 | 30·2 | 24·5 | 19·4 | 16·5 | 13·0 | 10·4 | 8·9 |
| Mean absolute deviation | 0·8 | 0·7 | 0·8 | 0·5 | 0·3 | 0·7 | 0·4 | 0·4 | 0·5 | 0·3 | 0·3 | 0·6 | 0·5 |

Fig. 1 shows the mean decomposition curve for $HbO_2$ under the action of acetic acid. The figure shows that the maximum decomposition rate of haemoglobin from



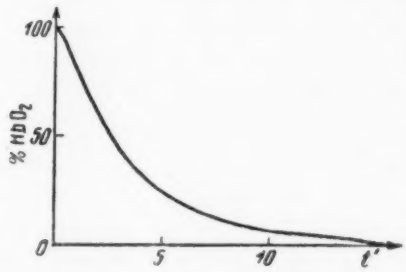




FIG. 1. Decomposition curve of $HbO_2$ under the action of 0·1 *N* $CH_3COOH$; $t = 25°C$.
FIG. 2. Decomposition curve of $HbO_2$ under the action of 0·025 *N* NaOH; $t = 25°C$.

healthy subjects under the experimental conditions chosen occurred at the 3rd minute. The decomposition curve is S-shaped, but at the same time the relative reaction rate

did not depend on the concentration of $HbO_2$, which is characteristic of first order reactions. Variations in the decomposition rate between individual subjects were very small and within the limits of experimental error.

A study of the kinetics of alkali (NaOH) decomposition of $HbO_2$ from healthy human subjects (11 male and 7 female) aged between 17 and 55 years showed that this decomposition curve was also very regular, no differences due to age or sex being observed. The experiments were at 25°C, and the NaOH concentration was 0·025 *N*. Table 2 gives the mean results of these experiments.

TABLE 2

| Time (min) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % HbO remaining at time $t$ $(100 - C)$ | 79·8 | 59·5 | 43·7 | 32·2 | 24·3 | 18·0 | 13·7 | 10·1 | 7·6 | 5·9 | 4·5 | 3·4 | 2·7 |
| Absolute mean deviations | 1·1 | 0·9 | 0·5 | 0·8 | 0·8 | 0·5 | 0·3 | 0·6 | 0·4 | 0·5 | 0·8 | 0·4 | 0·2 |

Fig. 2 shows the mean curve for the decomposition under the action of alkali of oxyhaemoglobin from a number od normal subjects. A comparison of Figs. 1 and 2 shows that the decomposition curves of $HbO_2$ under the action of acid and alkali differed substantially for the same reaction time.

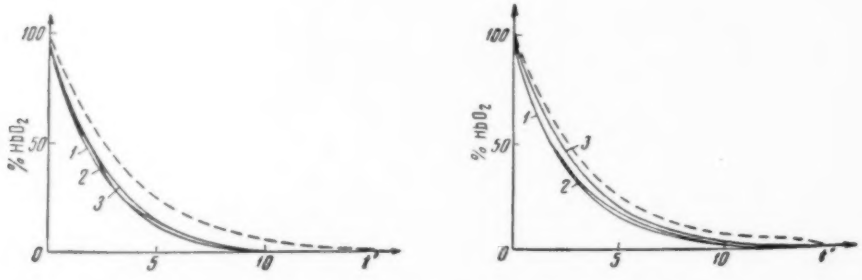



FIG. 3. Decomposition curves of $HbO_2$ under the action of alkali (0·025 *N* NaOH). (1) Basedow's disease, (2) Basedow's disease, (3) cardiovascular insufficiency. Broken line — decomposition curve of $HbO_2$ for healthy subject.

FIG. 4. Decomposition curves of $HbO_2$ under the action of alkali (0·025 *N* NaOH). (1) atrophic cirrhosis of the liver, (2) cirrhosis of the liver, (3) acute nephritis. Broken line — decomposition curve of $HbO_2$ for healthy subjects.

It was of interest to examine the deviations from the normal course of $HbO_2$ decomposition in some cases of disease. The oxyhaemoglobin decomposition studies on patients were carried out by the same methods and under the same conditions as those used for healthy subjects. In some pathological cases, the stability of haemoglobin to alkali was markedly reduced. Moreover, the curve differed sharply from the normal; as will be seen from Figs. 3 and 4 and Table 2, the differences cannot be ascribed to

experimental error. In some pathological cases (cirrhosis of the liver, acute nephritis) the total decomposition time was unchanged; in other cases (Basedow's disease, third stage cardiovascular insufficiency) it was slightly reduced, but in all these cases the decomposition half-life was sharply reduced; thus, it was $3\pm0{\cdot}1$ min in normal subjects but in subjects with diseases it was reduced to $2\pm0{\cdot}1$ min.

Fig. 3 shows oxyhaemoglobin decomposition curves of three patients in which the total reaction time was accelerated. Fig. 4 shows decomposition curves of $HbO_2$ in three pathological cases in which the decomposition time was unchanged. The dotted line on both figures indicates the $HbO_2$ decomposition curve for a healthy subject.

In the present work we did not aim at covering a wide range of diseases or at a detailed study of particular pathological cases. The examples given show that in pathological states a sharp acceleration in the decomposition is observed at the beginning of the reaction, and a retardation at the end. This gives grounds for suggesting the presence of qualitative changes in the composition of haemoglobin in patients with some diseases.

The results of haemoglobin decomposition studies under the action of alkali (NaOH) for the foetus, and for new-born and adult subjects are given in Fig. 5. The curves were obtained at the same blood concentration, alkali concentration (0·2 *N*) and

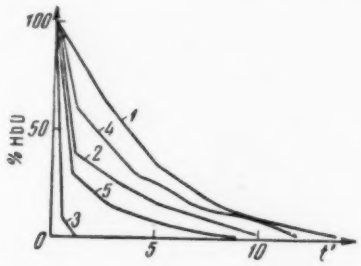



FIG. 5. Decomposition curves of $HbO_2$ under action of 0·2 *N* NaOH (1) embryo, (2) new-born subject, (3) adult human subject, (4) rabbit, (5) mixture of 50% human $HbO_2$ and 50% rabbit $HbO_2$.

temperature (25°C). These experiments showed that the alkali stability of foetal haemoglobin differed greatly from that of haemoglobin from adult subjects. For adult $HbO_2$ the reaction was complete in one minute under the conditions chosen, but for foetal blood the reaction time extended to over 12 minutes.

The $HbO_2$ curve for the new-born baby is very characteristic. It occupies, as it were, an intermediate position between the adult and the foetal curves, with a sharp inflection at the point corresponding to a reaction time of one minute. The inflection may possibly be explained by the presence in new-born blood of two kinds of haemoglobin differing in their stabilities to alkali. In a model experiment carried out on a mixture (50:50) of rabbit and human blood it was shown that for a mixture of two

haemoglobins differing markedly in stability a sharp inflection occurred after one minute. If it is assumed that new-born haemoglobin consists of two fractions, then it may be supposed that for the experiment illustrated in Fig. 5 the quantity of alkali-stable haemoglobin in the new-born baby examined amounts to 50 per cent of the total $HbO_2$.

The peculiar course of the curves for the alkali decomposition of new-born haemoglobin is of interest in growth physiology and in practical pediatrics, because of the possibility of evaluating the degree of development of the blood-producing apparatus in the new-born.

Studies with acetic acid showed that there was no marked difference between foetal, new-born or adult haemoglobin in regard to this reagent. This confirms results obtained by other authors [2].

## SUMMARY

(1) The kinetics of $HbO_2$ decomposition and qualitative changes in its composition were followed by a photoelectric method.

(2) The curves for acid and alkali decomposition of $HbO_2$ from adult subjects were very regular; they were not related to age or sex.

(3) The decomposition curves obtained by the action of alkali on $HbO_2$ from pathological subjects differed markedly from those for $HbO_2$ from healthy subjects.

(4) Oxyhaemoglobin from a new-born baby decomposed under the action of alkali as a mixture of two kinds of haemoglobin that differed sharply in stability.

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*Translated by* E. HAYES

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# STRUCTURAL MOBILITY AND AUTO-REGULATION OF CELL PROCESSES*

L. G. VIEZE and G. M. FRANK

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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A RECURRENT theme of discussion in our investigations has been the role of the mobility of cellular structures with change in the functional state [1–3]. The present communication reports some new facts and ideas on this question.

It is a matter not so much of specialized apparatus for movement such as muscular contractions, amaeboid movement, etc. but rather mobility not directly related to execution of mechanical work. In investigations in our laboratory, these particular manifestations of mobility have been distinctly revealed in study of the origin and spread of excitation along a nerve fibre, where figuratively speaking the "micromobility" of the structure is such a manifestation [1, 4, 5]. It was of importance to conduct a comprehensive study of how general and widespread is this phenomenon. For this reason we resorted to the interference technique first used by Kaiushin and Liudkovskaia [6] for observing the surface of the nerve fibre. This technique has been described [6] and we shall therefore confine ourselves to the reminder that passage of nerve impulses along a nerve fibre is accompanied, on observation of the nerve surface in the interference microscope, by "derangement" of the interference pattern through displacement of the interference bands which in turn is due to the micro-displacement of the surface of the conducting nerve. Insufficient attention has been paid in the past to the fact that in addition to the structural wave in response to a stimulating impulse it is possible in many cases to observe spontaneous, relatively-slow mobility of the structure of the isolated nerve [6]. We have coined the metaphoric expression "structural respiration" of the nerve to describe this process.

Such a type of structural fluctuation was discovered by us with direct measurement of the visco-elastic properties of nerves [7]. The technique applied made it possible to record, in time, the width of the hysteresis loop with continuous semi-twisting and unwinding of the living nerve. As long as the nerve remains alive, this value undergoes continuous variation. These findings should be compared with the recently-discovered fluctuation in the threshold value of excitation of the nerve fibre [8].

Since these results were obtained, other workers in our laboratory have been able to demonstrate the presence of continuous, functional mobility by other methods. Aladzhalova [9, 10] was able to detect ultra or infra-slow variations in the potential

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of the direct current in parts of the central nervous system of higher animals and on the most elementary specimens. Snezhko [11] by the method of polarographic recording also discovered continuous variations in the free oxygen tension in the most diverse tissue not associated in many cases with blood circulation. Both electrical and oxygen rhythms have a frequency of a few fluctuations per minute.

It was important to find out whether these rhythmical electrical and metabolic processes are in turn linked with the structural micromobility in these specimens.

The distinct, slow rhythms of variations in the direct current potential and also the variation in the level of oxygen uptake can be found for a specimen which keeps well — such as the sub-oesophageal ganglion of the cricket. This specimen was investigated by means of the interference technique mentioned above. We would recall that recording of the interference patterns was by projection of the narrow portions of the interference bands on to a moving photographic film through a narrow slit. With the specimen immobile, this recording system forms strictly parallel lines on the film. Fig. 1 depicts the interference pattern obtained from the surface of the cricket

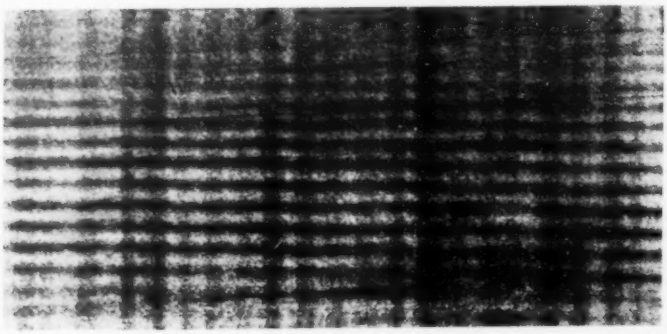

FIG. 1. Interference pattern from the surface of cricket sub-oesophageal ganglion, one day after preparation.

ganglion 24 hr after dissection. The parallel nature of the bands suggests the immobility of the structure. Structural displacement leads to displacement of the interference bands and then, in relation to the degree of this shift, either to deflexions or breaks in the parallel lines. With rhythmical mobility these deflexions have a wave-like character and for violent or quickly-developing structural activity bizarre swirls are seen in place of the picture of steady parallel bands.

On observing the surface of the dissected cricket suboesophageal ganglion while still alive, it is possible to note continuous, rhythmical structural shifts which develop more or less rapidly. These phenomena gradually subside and are no longer found, for example, after keeping the tissues for 24 hr (Fig. 1).

As Fig. 2 shows, in the living ganglion well-marked rhythmical structural variations with a wave-like character and periodicity of the order of one fluctuation per sec are observed.

Electrical or chemical treatment of the ganglion apparently alters the character of this spontaneous rhythm. However, the fact that the size of the ganglion is geometrically so small always creates the danger that additional treatment will produce purely mechanical displacement effects and at this stage of the experiments we were not able to obtain sufficiently reliable results on the character of the response of the ganglion to the electrical stimulus.

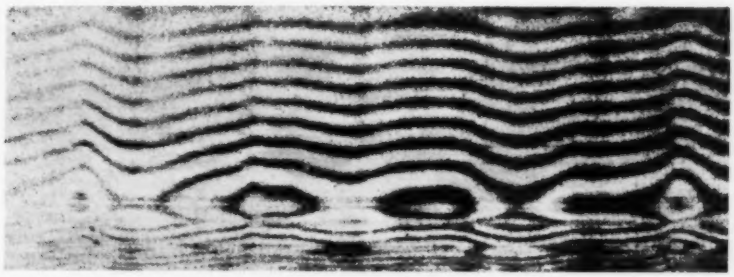

FIG. 2. Similar, but livening, preparation showing rhythmical variations in the bonds.

For this reason we focused our attention on a different specimen — the central nervous system of the frog.

The entire central nervous system i.e. brain and spine of the frog was dissected out and placed in a polymethylmethacrylate humid chamber in such a manner that

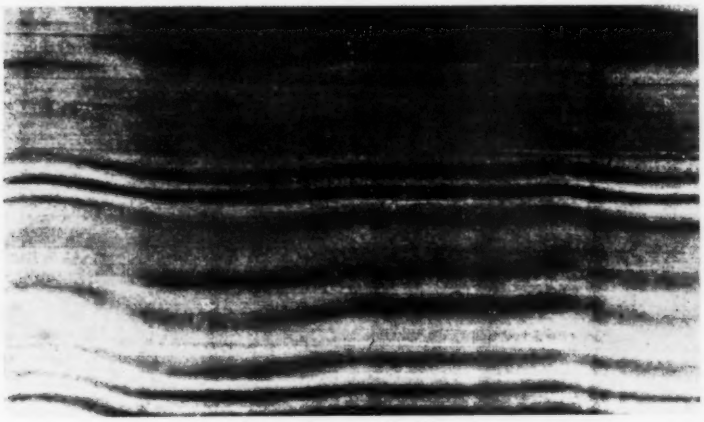

FIG. 3. Interference pattern from frog spine showing rhythmical variations.

middle part of the spine or the region nearer the caudal section was in the field of vision of the interference microscope and the stimulating electrodes used for pain stimulation were applied to the brain. The spine of the frog also displayed spontaneous structural mobility (Fig. 3).

However, this spontaneous mobility was not so clearly expressed as for the cricket ganglion. The amplitude of the variations was, as a rule, low although here the shift in the bands was of a wave-like character.

We performed various control tests to satisfy ourselves that this rhythmical mobility was unconnected with any of the capillary phenomena observed in the humid chamber: the phenomenon of drying, redistribution of water vapour, etc. For this purpose, similar observations were made initially on the specimen, after keeping it for 24 hr at room temperature, and also after inactivation in saline at 56°C. In all cases, the

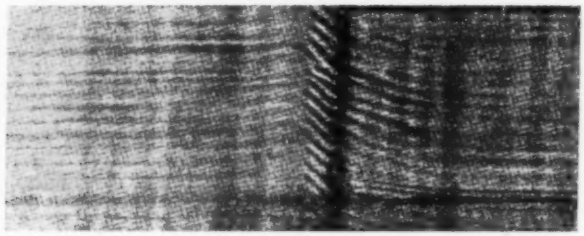

FIG. 4. Interference bonds seen in frog spine after the application of a current.

picture was radically changed. In the vast majority of cases, complete immobility set in and the system of interference bands had a very steady character. Further explanation is required of the small shifts noted in certain isolated instances and a solution of the problem as to whether they were residual functional activity or, in the pure form, artefacts connected with the liquefaction of the substrate of the brain which is sometimes observed and perhaps with the ready discharge of this liquified fraction from the damaged spine on dissection and subsequent manipulation.

Two types of structural mobility of the spine were observed in response to electrical stimulation of the brain. On the one hand, in response to a single impulse or single switch on or off of the direct stimulating current there was a rapid transient shift of the bands with a wave-like shift of the entire interference pattern (Fig. 4). On the other hand, as a rule, the effect of a series of electrical pulses was to produce within 2–3 sec of the start of stimulation (and sometimes even sooner) a "swing" as it were of the structural picture with intensification and modification of the spontaneous movements and a sharp increase in the amplitude with a quiescent state sometimes only 10–30 sec after ending stimulation. This quiescence came about gradually with retention of the long tail of the disturbed rhythm and differing appreciably from the resting state (Fig. 5).

The storage of the preparation for a day at room temperature, or half an hour in saline at 56°C., stopped the structural response to electrical stimulation. The "swing" of the structure and the emergence of the rhythm as a result of strong electrical pulses were never observed. In individual cases, it was possible to record the original appearance of the break in the interference lines in response to switching on of the direct current. However, the artefact was readily distinguished from the reaction of the live

preparation. As has already been stated in the normal functional state wave-like, i.e. reversible, shifts occur. The displacement in individual cases in the inactivated preparation, obviously due to the unsuccessful application of the electrodes, was the result of the cataphoretic mobility of the fluid moistening the preparation and had the character of a unilateral break, a unilateral shift indicating the mechanical impetus without the ability of the system to return to its original state.

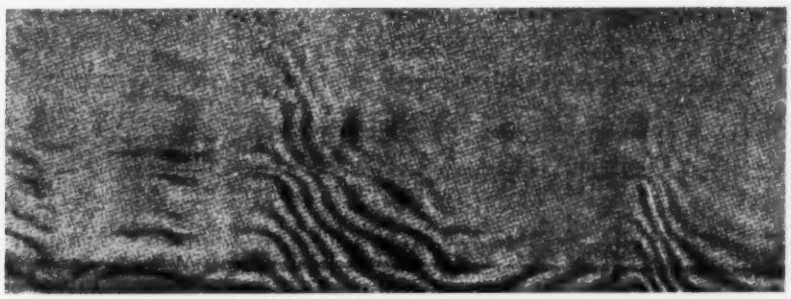

FIG. 5. As Fig. 4, but after a series of stimuli.

The data obtained were not unexpected and do not conflict with the concepts on the structural organization of the living substrate which in one form or another exhibits structural mobility. Indeed, the flow of protoplasm is an extremely widespread phenomenon. As stated above, we discovered a continuous change in the visco-elastic properties. The light scattering effect of a living substrate is also not a constant value, not to mention the electrical potentials and levels of free oxygen.

Thus, the facts presented in this communication merely add to our ideas on the universal phenomenon of structural mobility, necessary for the maintenance of vital processes. In discussing the importance of mobility itself, we must return to the point of view developed by us during the course of the last two years, that structural mobility is a necessary link in the automatic regulation and self-adjustment of the living system to a definite level of vital activity [1, 3]. The purely speculative aspects of this hypothesis are based on the fact that in preserving vital activity a strictly definite structure is necessary, not only intracellularly but also in the macromolecule structure ensuring the orderly pattern and coordination of the course of a considerable number of chemical, metabolic processes. On the other hand, only when these proceses have a continuous course is the structural organization maintained. A typical feed-back system is present here. Disturbances and modifications in the structure change the course of the chemical process while this process in turn affects the structural organization. In living cells not only must the degree of perfection of the automatic regulatory processes be remarkably high, but there must also be what is known in modern techniques as self-tuning to the most advantageous system of work. As is known, technically this is best achieved by use of pulse or relay methods for the self-righting mechanisms, providing a certain rhythmical pulsation of the activity of the entire feed-back system.

We imagine this general and universal phenomenon of structural mobility with the associated interaction of the rhythm of this mobility with the rhythms of the chemical metabolic processes to be a possible link in the auto-regulatory mechanism of the living system. The mechanism has been produced during millions of years of evolution of living matter and modern techniques in the production of self-tuning and self-regulating pulse systems are starting to model themselves on it.

In conclusion, certain assumptions, requiring confirmation and verification in the future, can be made on the specific mechanisms of regulation.

In turning to the study of the mobility of objects not specialized for mechanical work and not containing contractile fibres etc., it is natural to assume that this mobility is in accord with the simple principle governing amoeboid movement, by displacement and flow of protoplasm in undifferentiated cells. It is known that in such cases a major role is played by the reversible transition of individual areas of protoplasm from sol into gel and gel into sol. If it be imagined that in the individual segments of the cell, in groups of cells or in nerve conductors there is continuous formation of islands of gelatinization followed by liquefaction, it would be natural to expect that local forces of tension and deformation would arise, sufficient to produce mechanical displacement by a fraction of a micron.

If ionic mobility in the loops of protoplasmatic protein gel shows virtually no difference from the mobility in the sol — due to considerable pore diameter in the former —, this cannot be said of the large molecular complexes formed by the processes of synthesis and moved by the forces of diffusion or active transfer of a new type of transporting enzyme. The formation of a gel may be a special trap for the movement of the complex. Restrictions on movement cause different concentration gradients not allowing them to become equalized and lead to a block of the enzyme working system preventing, figuratively speaking, the products of the enzyme system from being swept away and not allowing the free flow of the chemical conveyor belt for further activity.

This is not to mention that the flux of the protoplasm and cellular mobility are the rudiments of a particular transport system ensuring and regulating the movement of substances in the cell. If we imagine a given cycle of chemical activity connected with formation or degradation of a relatively large molecular complex and in turn connected at a certain stage with the uptake of oxygen, the delay in freeing the working structure from the products formed will also be connected with delay in the uptake of oxygen. We are aware of two facts of great importance in the transition of sol to gel and vice versa. On the one hand, it is a matter of ionic screening effects of the electrolytes in relation to the forces of cohesion between the molecular complexes, which has been convincingly demonstrated and calculated in great detail by Kirkwood *et al* [12]. On the other hand, the data of Kun, published only in the form of a recent abstract, indicated that the shifts in the redox potential with changed pH in model systems lead to gelatinization or conversely to sol formation. It should be added that the various enzyme systems, as is known, require different levels and ranges of the redox potential. Thus, we have the whole system of interdependent processes and

phenomena. The uptake of oxygen and the variation in the level of oxygen uptake are not without their importance in the redox potential of the system. The latter change the degree of aggregation or gel formation. Gel formation shifts the balance in the transfer and rates of distribution of substances which in turn affect the rate of oxygen uptake. Changes in the redox potential contribute to a greater or lesser degree to the inclusion or exclusion of a particular enzyme system. The associated liberation or binding of the ions of the electrolytes in turn affects the degree and character of the interaction of the protein macromolecules.

It should be added that these processes of transition from sol into gel and vice versa, linked with the appearance of definite forces of cohesion, may affect the level of the functional activity of the enzymes entering the structure of the systems undergoing change. Of course, at this stage, this assumption is only of a purely qualitative character.

VOL. 5 1960

## SUMMARY

(1) A further study has been made of the micromobility of cellular structures in the processes of vital activity, in particular, excitation using the interference technique.

(2) Rhythmical wave-like structural fluctuations were noted on investigating both the surface of an isolated suboesophageal ganglion of the cricket and the spine, dissected together with the brain of the frog (Figs. 2, 3). Specimens inactivated at 56°C. or kept for a day at room temperature no longer displayed mobility of structure (Fig. 1). On recording the interference bands, a parallel row of straight lines was obtained.

(3) On application of a pain stimulation by means of an electric current to the frog brain, in the spine a wave-like shift was observed in the interference pattern (Fig. 4) with increased amplitude of the variations and an intensified "swing" (Fig. 5).

(4) The observations are compared with certain data in the literature and the assumption made that the structural mobility is a manifestation of feed-back between the chemical metabolic processes and a definite structural organization. It is supposed that this mechanism is a fundamental link in the automatic regulation and selfadjustment of the living system to a definite level of vital activity.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# ACTION OF ULTRA-VIOLET RADIATION ON SINGLE NERVE FIBRES*

R. G. LIUDKOVSKAIA and L. P. KAIUSHIN

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
5
196

LIVING cells are very sensitive to ultra-violet radiation. This general property is connected with the part played in the basic cellular structure by protein which has maximal absorption in the region of 275–280 mμ and by nucleoprotein which has maximal absorption in the region of 260 mμ. The importance of structural proteins and of nucleoproteins for the viability of cells is beyond doubt. However, it is not clear what role these materials play in the processes of excitation and conduction in nerve fibres.

Irradiation of a nerve fibre with monochromatic ultra-violet light affects solely the protein and nucleoprotein. These substances are destroyed when illuminated with ultra-violet light, leading in the end to the loss of excitability. Examination of the electrical activity of a nerve fibre makes it possible to follow changes in excitability and conductivity at the time of irradiation, and by so doing to determine the kinetics of the process.

The chemical and structural heterogeneity of a nerve fibre leads to different absorption of ultra-violet light by the surface and central parts of an axon. This makes it possible to demonstrate the role of these parts of the nerve in the formation the action potential (or spike) and in the excitation process.

In earlier investigations [1, 2, 3] it was established that monochromatic radiation with a wavelength between 260 mμ and 280 mμ has a strongly injurious effect on myelinated nerve fibres. At the same time there was evidence of considerably heightened sensitivity at the nodes of Ranvier, in comparison with the internodal region; the rate of rise of the excitation threshold served as a measure of the effectiveness of the ultra-violet radiation in these experiments. Subsequently an attempt was made at the photochemical analysis of the wave of excitation by combining brief flashes of

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ultra-violet light with successive phases of the action current [4]. There was evidence (from the rate of rise of the threshold) that the ultra-violet light flashes had considerable photochemical effects at the moment of excitation and also immediately before the start of the action potential, but had less effect if they occurred immediately after the end of the spike. There has been no study hitherto of the effect of ultra-violet light on the non-medullated giant axon.

In the present work we have studied and compared the effect of monochromatic ultra-violet light on the action potential of single myelinated fibres of the frog and of giant (non-medullated) axons of the squid (*Ommatostrephes sloanei pacificus*). A comparative study of these objects should help to reveal the role of nucleic acids and proteins in the nerve membrane and axoplasm in promoting the general electric behaviour and the separate phases of that behaviour.

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Our study did not entail a quantitative examination of the problem, or a detailed calculation of the absortion of light by a nerve fibre. In the first instance it was more important to study the similarities and differences between the electrophysiological responses of excited nerve fibres of different types under the photochemical action of ultra-violet light.

## METHODS

A single motor fibre of the frog's sciatic nerve was placed in a chamber, the bottom of which consisted of two quartz plates separated by an air gap of about 1 mm. An internodal section of the fibre was placed across this gap and on each side of the gap the nerve lay in Ringer solution into which dipped the platinum detecting electrodes. The stimulating electrodes were 1–5 cm from the detecting ones. The chamber, containing the preparation, was clamped to the stage of a type MUF-3 ultra-violet microscope. A similar chamber was used to study the squid giant axon. The axons, carefully separated from the nerve, were between 200 and 400 $\mu$ thick.

The source of ultra-violet light was a type SVDSh-1000, or type SVD-120. The light beam passed through a heat filter and fell on a section of the nerve fibre in the region of one of the detecting electrodes. The electrodes were connected to a constant current amplifier of high input resistance, and thence to a type ENO-1 oscillograph with a screen from which the action currents could be photographed. The nerve was stimulated with rectangular pulses of $10^{-4}$ sec duration, applied at a frequency of 1–50 per sec. We used various ultra-violet filters.

The light beam was focussed on to an area of 0·1 to 0·2 mm$^2$ and the preparation could be arranged with this spot on a node of Ranvier or on an internodal part of the fibre. However, the change in action potential when one node was irradiated was very small, and the effect was considered to be, for the most part, a raising of the threshold. Eventually we increased the irradiated region of the fibre until it was 4–5 mm long, so irradiating two or three nodes and increasing the effectiveness of the radiation.

In experiments with pulsed illumination the excitation of the nerve was synchronized with the ultra-violet light flash (which was of 0·005 sec duration); the flash could be made either coincident or non-coincident with a particular phase of the action current. The frequency of the flashes was 100 per sec.

## THE ACTION OF ULTRA-VIOLET LIGHT ON MYELINATED FIBRE

*Ultra-violet and visible light* (but excluding infra-red) of high intensity (from a type SVDSh-1000 lamp) caused a rapid rise of the action potential. This increase began immediately after the light was switched on and lasted for 1–2 min (if the irradiation was continued); it was accompanied by some fall in the threshold. Such an increase in the action potential was, as a rule, observed in a fibre with a constant excitability. In fibres which functioned irregularly, irradiation caused an immediate diminution of the spike; a brief rise in the action potential after 2–3 min alternated with the fall, and a steady rise of the threshold.

The size of the irradiated region influenced the survival time of the fibre. Local irradiation with, and absorption of, very intense light caused a comparatively slow change in the conducting properties of the fibre. An increase in the area of the luminous spot, even with a reduction of light intensity, led to a considerably heightened effectiveness of irradiation. If, in the case of intense irradiation, the fibre retained excitability for about 10 min, then if a larger area was irradiated the action potential fell within 10 sec.

It might be thought that the rise in the amplitude of the action current was connected with a rise in temperature of the fibre during the exposure. However, neither the ultra-violet nor the visible part of the spectrum separately caused a rise in the spike amplitude.

*Ultra-violet light in the wavelength range 240–400 mμ* had a powerful effect on the rising phase of the action current, causing a diminution of the gradient $\Delta v_b = A/(\Delta t)$, where $A$ is the amplitude of the action current and $\Delta t$ is time it takes to reach its maximum value. In some cases the first result seemed to be a brief increase in the gradient of the rising phase, with a subsequent decrease. The falling phase either did not change or else its gradient decreased rather more slowly than that of the rising phase.

*The narrow spectral region of wavelength 320–390 mμ* was nearly without effect on the size of the action current. Changes in the rising and falling phases during the irradiation made them fluctuate about their initial values. However, if the irradiated preparation consisted of two fibres, then changes in some properties (e.g. threshold, conduction velocity) were noticeable. A single-peaked action current was divided between two identical fibres during irradiation, and the same strength of stimulus developed a double-peaked potential. This appeared to be a consequence of different changes occurring in the velocity of conduction and excitation in each fibre (Fig. 1). The resistance of the fibre decreased and this cut down the rise of the associated excitation current.

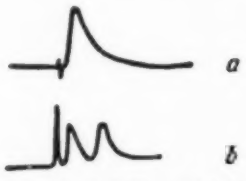


FIG. 1. Action currents in two myelinated fibres: (a) before irradiation; (b) during irradiation with light in the spectral region 320–390 mμ.

*Ultra-violet light in the wavelength range 270–380 mμ* had a very strong effect on the electrical behaviour of the fibre. It caused sudden falls in size of the action current and of the gradients of the rising and falling phases. The excitability of the fibre and the action potential both disappeared within 20–40 sec. The activity of this part of the spectrum was connected with the action of light of wavelength 275–280 mμ, which is absorbed by protein.

*The spectral region of wavelengths from 250 to 285 mμ* was less effective than light of wavelength 280 mμ, even though the intensity of light at 280 mμ was the same in both cases. The gradients of the rising and falling phases changed much less under the combined action of light of wavelengths 260 and 280 mμ than under the action of light of 280 mμ alone (Fig. 2).

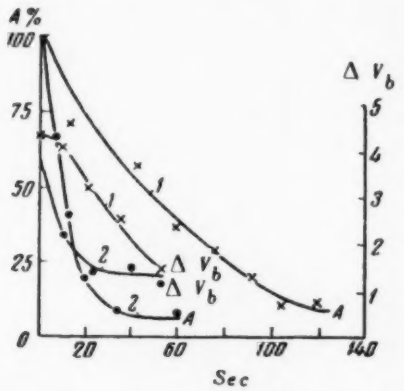


FIG. 2. Changes in amplitude and gradient of the rising phase of the action current during irradiation of a myelinated fibre. *A*: change in amplitude in per cent. $\Delta V_b$: change in gradient of the rising phase. Wavelength of the light: *1* — 260 mμ and 280 mμ together; *2* — 280 mμ only.

In our work, as in that of von Muralt and Stämpfli [4], coincidence of the action current with a flash of ultra-violet light illuminating the fibre led to a more rapid loss of excitability than in the case when the flash occurred in the interval between pulses. On stimulating the irradiated nerve without interruption with pulses given at a frequency of 50–100 per sec, excitability was lost two to three times quicker than in the case of single stimulation. After terminating the stimulus, the action current partially recovered, despite the continuing irradiation. These facts serve to demonstrate that ultra-violet light acts on the excited system in which an essential role is played by structural proteins and nucleic acid.

## THE ACTION OF ULTRA-VIOLET LIGHT ON THE SQUID GIANT AXON

Experiments with the giant axon differed from the proceeding ones in that the intensity of the ultra-violet light (emitted by an SVD-120A lamp) was less and the spike was in most cases recorded biphasically. This technique was necessary because

the action of ultra-violet light on the excited part of the fibre adjacent to a depolarized section led to a very rapid loss of excitability. In experiments with monophasic recording the results in two cases were similar.

In the giant axon under the influence of ultra-violet light there were changes in the action current, the gradient of the rising phase and the threshold of excitability. A fall in the amplitude of the action current appeared to be a typical reaction to irradiation. However, light of 270–380 mμ in some cases caused a rise in this amplitude (in distinction from the case of myelinated nerve); and the increased amplitude remained constant for some minutes. Such an increase of amplitude occurred in 25–30 per cent of the preparations examined. Very characteristic changes were observed in the shape and gradient of the rising phase. The gradient of this phase changed irregularly, but chiefly by an extension of its lower part; the rate of rise of the upper part of the spike remained unchanged. In the clearest cases sharp breaks could be seen in the rising phase of the action current, these breaks occurring near the top (Fig. 3, *3* and *4*).

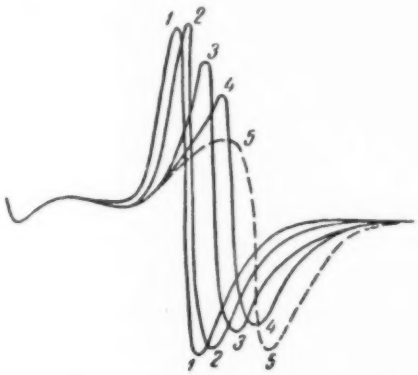


FIG. 3. Successive action currents in the squid giant axon during irradiation with light of 250–285 mμ. *1*: control. *2*: after 3 min 10 sec. *3*: after 4 min 30 sec. *4*: after 4 min 40 sec. *5*: after 4 min 45 sec.

In the subsequent stages the size of the action current decreased abruptly (Fig. 3, *5*) and quickly appeared like a monophasic current. Changes in the slope of the rising phase and in the size of the spike were found to be closely associated (Fig. 4). During irradiation there was a gradual rise in the excitability threshold which seemed to be connected with a lengthening of the latent period.

The sensitivity of the axon to different spectral regions was examined. As a criterion of the effectiveness of the action of light we took the time between irradiation and the disappearance of excitability. In the case of biphasic recording this time could be determined from the transition between the biphasic appearance of the current and the monophasic diminishing potential. The most rapid disappearance of excitability and fall in size of the action current occurred on irradiation with light of 275–280 mμ: the combined action of light of wavelength 275–280 mμ and of wavelength 260 mμ

was less effective (Fig. 4). It appeared that light of 260 mμ yielded products which were protective chromophoric substances, absorbing light of wavelength 280 mμ.

Light of wavelength greater than 300 mμ appeared to have no effect on the electrical behaviour, or on the functional characteristics of the axon. It is known that the threshold for photochemical decomposition of pure proteins lies at about 300 mμ; i.e. the reaction can proceed if the energy of the absorbed quanta exceeds 95 Kcal/mole [5].

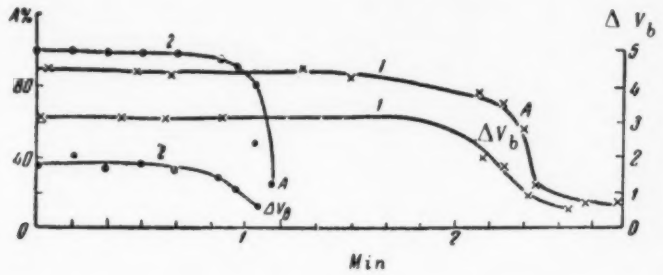


FIG. 4. Changes in gradient of the rising phase and amplitude of the action current during irradiation of squid giant axon. *A*: change in amplitude in per cent. $\Delta_{V_b}$: change in gradient of the rising phase. Wavelength of the light: *1* — 260 mμ and 280 mμ together; *2* — 280 mμ only.

The effect of the action of ultra-violet light on the nerve seemed at first to be reversible. Thus if the stimulation was stopped but the irradiation was continued, the size of the amplitude was restored.

The giant axon was more sensitive than myelinated nerve fibre to the action of ultra-violet light; this was because of the larger quantity of absorbing material. The injurious action of the light on a fibre surrounded by a myelinated membrane grew with increase in size of the illuminated fibre. If it is considered that the lipid partion of the membrane took practically no part in the general absorption at 275 mμ [6], then the protein in the nerve was the principal absorbing agent.

The most injurious ultra-violet light appeared to be that of wavelength 275–280 mμ. The chromophores which absorb most powerfully in this region contain the amino-acids tyrosine and phenylalanine. Precisely these amino-acids are contained in noticeable quantities in the protoplasm of nerve fibre [7]; but the cyclic amino-acids are not found in the axon membrane in the quantities which occur in the protoplasm. For this reason it can be concluded that ultra-violet light acted on the protein in protoplasm.

We may also suppose that the primary photochemical action occurred in the protein of protoplasm but that this process resulted in the formation of decomposition products which could bring about changes in the permeability of the membrane, leading to changes in the amplitude of the action current.

Growth of the spike amplitude, observed in the ultra-violet irradiated giant axon, was also seen as a result of X-irradiation [8].

We do not have sufficient data to analyse the effect on the excitability of the nerve of ultra-violet and visible light acting together. But from our data it follows that the role of nucleic compounds (which absorb in the region of 260 mμ) is relatively less important than that of proteins (which absorb in the region of 280 mμ).

## SUMMARY

(1) A study was made of the effect of ultra-violet light of various wavelengths in the range 240–400 mμ on the action currents in myelinated nerve fibres of the frog and non-medullated giant axons of the squid. The greatest effect on the electrophysiological characteristics of the fibres was produced by the spectral region of wavelength about 280 mμ which is absorbed by proteins.

(2) The primary photochemical reaction occurred in proteins of the nerve fibre protoplasm. It is supposed that products of photochemical decomposition of proteins affected the permeability of the membrane and that this led to changes in the amplitude of the action current.

(3) It was found that the effect of visible and ultra-violet light acting together differed from the effect of each acting in isolation.

(4) Ultra-violet light of wavelength 280 mμ had a very great effect on excited nerve fibres. But this effect was decreased if the stimulation frequency was low or if illumination occurred as a flash in the interval between stimuli. Our data are in agreement with the results of Ungar, Aschheim, Psychoyos, and Romano [9] who showed that decomposition of the structural proteins of the nerve takes place during excitation.

(5) The results of irradiating with ultra-violet light or with X-rays [8] lead to parallel changes in the action current.

*Translated by* D. ROSEN

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# THE EFFECT OF FRACTIONATED DOSES OF X-IRRADIATION ON THE CAPACITY OF PLANTS TO RESUME GROWTH*

I. M. VASIL'EV and B. G. ZHUKOV

Institute of Bio-physics, U.S.S.R. Academy of Sciences, Moscow

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VOL. 5 1960

VASIL'EV [1] has remarked that the growth of wheat, maize, etc. inhibited by X-irradiation may be resumed after a time, particularly in the light. The resumption of growth, however, depends to a large extent on the dose of irradiation. Thus, after air-dried seeds of winter wheat 599 were given 50 kr, root growth in almost all the plants was resumed after about $1\frac{1}{2}$ weeks. After 100 kr, renewed root growth began later and only in a few plants.

In the present investigation we have made a further study of this question. The experiments were made in the following way. Seeds of winter wheat 599, selected for size, were soaked in tap-water and germinated in a luminostat under constant illumination and at a temperature of $23 \pm 2°C$. Uniformly germinated seedlings when one-day old were set out in groups of 100 on polymethyl methacrylate sieves over Koch dishes completely filled with Knop's nutritive solution and covered with bell-jars to produce a vapour-saturated atmosphere above the plants, the whole being then replaced in the luminostat. The seedlings were irradiated on the same day. One experiment included the following groups: (1) not irradiated; (2) irradiated once with 5 kr on the first day; (3) an initial 1 kr irradiation and the same amount on each of the next 4 days; (4) 500 r irradiation morning and evening during 5 days; (5) 5 kr on the fifth day. Another experiment included the same series with one addition; on the first day the plants received four doses of 250 r at 2-hour intervals and the same treatment was given on the next 4 days. Thus, with the exception of those not irradiated at all, the plants in each series received 5 kr irradiation, the difference being only in its fractional subdivision. The X-ray apparatus was always the same: L RUM-3, 165 kW, 15 mA, without a filter. Before irradiation the plants and sieve were removed from the Koch dish and placed in a shallow Petri dish without nutritive solution. After irradiation they were returned to the nutritive solution and replaced in the luminostat.

Fig. 1 shows the results of one of the experiments. The plants were photographed four week's after the initial irradiation. It can be seen that the inhibiting effect was greatest in the plants given the entire 5 kr irradiation on the first day. The more

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fractioned the dose, the less effect it had on the plants, the least effect of all being observed in the plants given 5 kr on the last, fifth day. In these plants the effect was still not fully apparent by the time they were photographed.

The result is not at all surprising. The more fractioned the total irradiation dose, the longer growth continued before being completely inhibited on receipt of a critical amount — approximately 2 kr for wheat, according to Vasil'ev's data [1].

FIG. 1. Winter wheat 599. (I) non-irradiated plant; (II) 1-day old plant given 5 kr irradiation; (III) 1-day old plant given 1 kr irradiation and 1 kr on each of 4 following days; (IV) 1-day old plant given 500 r irradiation morning and evening and the same on the next 4 days; (V) 5-day old plant given 5 kr irradiation on the 5th day.

More important is the following. After the plants which had had the most fractioned irradiation had completely ceased growth and their radicles had become covered with a mass of root hairs, then six days elapsed during which there were no visible changes in the external appearance of the plants. But after this period new roots began to appear from the root tips — from what had been the meristematic zone. They grew unusually rapidly, without branching, had no root hairs and resembled rapidly lengthening threads. When the photograph was taken almost all the plants of this series had resumed growth.

After a further two days some plants of the third series, which had received less fractional irradiation, also resumed growth but only in a few cases. None of the plants of the second series, given the total 5 kr irradiation on the first day, showed any sign of forming new roots at the time the photograph was taken.

But this series exhibited the following unusual phenomenon in a very marked form: $1\frac{1}{2}$ weeks after irradiation the leaves began to be covered with white, sweet-tasting granules due to exosmosis from the cells. The utilisation of these substances had been halted by the suppression of growth, but their passage from the grain still continued and they were being excreted by the plant in an effort, as it were, to get

rid of them. A similar exosmosis had previously been noticed by Vasil'ev [2] when four-day and six-day old wheat plants were irradiated and left for 1½–2 weeks in the light under conditions which favoured photosynthesis. As a result products of photosynthesis, in the form of sugar, were excreted on to the leaves where they crystallized. The same thing occurred in the present case, but not owing to photosynthesis since the shoot was still enclosed in the coleoptile, but owing to the hydrolytic action of the enzymes that make the reserve material of the endosperm available for the embryo. To a lesser extent the same phenomenon was also seen in plants of the third series, but it hardly occurred at all in those of the fourth. In brief, the more vigorous the growth of the plant the less material was found on the leaves.

A second experiment on a somewhat larger scale fully confirmed the results of the first. In this case, in the additional series of plants given 250 r irradiation four times on each of five successive days inhibition of growth was less than in the other fractional series, and root growth was already resumed on the 5th day after irradiation had ceased.

VOL. 5 1960

Thus, in the experiments described above we find very marked differences in the radio-sensitivity of plants given the same dose of irradiation but in different fractions. The most radio-resistant were plants receiving the most fractioned irradiation, the least radio-resistant were plants given the whole amount at once on the first day.

The varying radio-sensitivity of the plants cannot be explained by age differences since we are dealing here with root growth. The meristematic zone was alike in all the root tips and had the same typical features. In this respect roots differ from leaves, which grow only for a certain time and the meristem of which becomes less active with age and finally disappears altogether. The cause lies elsewhere: fractioned irradiation *ipso facto* produced less effect. Plants given a small dose insufficient to suppress growth were hardened for the next dose and became more resistant. In this respect, therefore, the effect of X-irradiation is like that of slight frost or drought, after which potentially frost- or drought-resistant plants acquire resistance. But, of course, there is a difference in principle between increased radio-resistance and frost- or drought-resistance, since all ionizing radiation, even in "hardening" doses, also causes many irreversible changes expressed particularly clearly in the alteration of inherited characteristics.

*Translated by* C. P. Dutt

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# EFFECT OF CHRONIC IRRADIATION ON DRIED RYE SEEDS*

L. P. BRESLAVETS

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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INVESTIGATIONS of the effect of X-rays on seeds and and fruits of various plants including dried rye grains were instituted by us as long ago as 1933. The investigations demonstrated stimulation of growth and development and a raised yield for some agricultural plants. We associated the stimulatory effect of irradiation on rye grains with definite cytological changes. Repeated experiments over a number of years have confirmed the correctness of our early work.

In the present work, we have investigated the results of prolonged irradiation of the seeds with weak sources of $^{60}$Co $\gamma$-rays. We placed the rye seeds at a distance of 50 cm from a weak source of 100 $\mu$c so that they received 0·53 r per hour and 341 r over 27 days†. This was a comparatively weak stimulus since repeated experiments with X-rays had shown that 1000 r was required to produce cytological changes in the cells of rye roots growing after irradiation. Another interesting aspect of these experiments was that chronic irradiation increased the rye yield, in trials on the small plots of the vegetable station of the Timiriazev Agricultural Academy, more than a single dose of 1000 r X-rays.

Table 1 gives the average data for 100 plants from sheaves taken at random. One spike from the main shoot of each plant was investigated.

TABLE 1

| Dose (r) | Length of spike (cm) | Weight of spike (g) | Number of seeds per ear | Weight of seeds of one ear | Length of grains (mm) |
|---|---|---|---|---|---|
| Controls | 9·0 ± 6·23 | 1·70 ± 0·63 | 40·1 ± 1·65 | 0·81 ± 0·046 | 8·6 ± 0·13 |
| 1000 r X-rays | 9·8 ± 0·28 | 1·12 ± 0·064 | 35·3 ± 1·57 | 0·87 ± 0·064 | 8·9 ± 0·15 |
| $^{60}$Co(341 r) | 10·1 ± 0·22 | 1·78 ± 0·063 | 43·8 ± 0·13 | 1·18 ± 0·058 | 9·2 ± 0·018 |

Crop analysis showed that the length of the ear and of each grain changed little in response to $\gamma$-irradiation, but that the weight of the seeds was increased quite con-

* Biofizika **5**: No. 1, 49–54, 1960.

† The radiation measurements and calculations were made by Prof. Ia. L. Shekhtman to whom we express our deep gratitude.

siderably, accounting for the increased weight of 1000 seeds — this is the criterion to which great importance is attached in agriculture (Table 2).

These experiments showed that while single doses of X-irradiation increased the weight of 1000 seeds by 21 per cent, prolonged γ-irradiation with one third this dose gave a 40 per cent increase.

*Cytological investigations*

After establishing the rise in the yield of summer rye due to the prolonged action of γ-rays, we undertook cytological investigations of the roots of seedlings produced from the grains irradiated with $^{60}Co$ for 27 days. This was aimed at determining a link between the stimulation of growth and development induced by chronic irradiation and the increased rapidity of cell division, which we had established for the acute action of X-rays (1000 r). For this purpose we calculated the number and percentage of dividing cells in 5 transverse sections of 8 control roots and of 7 roots produced from irradiated seeds.

VOL. 5 1960

TABLE 2. ACTION OF X-RAYS AND $^{60}Co$ γ-RAYS

| Dose (r) | Weight of 1000 seeds (g) | Increase as compared with control (%) |
|---|---|---|
| Controls | 21,270 | 100 |
| 1000 r | 25,880 | 121 |
| $^{60}Co$ (341 r) | 29,800 | 140 |

To ensure that the cells counted were of the same age, for calculation purposes we used sections from the site at which the root undergoes differentiation into the central cylinder and periblem. In this way, we were able to observe the same phase of development with a fair degree of certainty. In order to arrive at the percentage of dividing cells, it was necessary to count the total number of cells in the preparation. This presented no particular difficulties for rye since the cells are arranged in remarkably well-ordered layers. As experience has shown, it was sufficient to take five sections from each plant, by taking, in most cases, only the primary root for fixation, whereby it is possible to obtain data on a definite number of plants. We made a painstaking estimation of the number of dividing cells in the control, unirradiated plants since experience had shown large variations in this number. In order to avoid overloading this paper with tables, we shall only give two, showing the total cell count and the number of dividing cells for the controls and irradiated seedlings (Tables 3 and 4).

Averaging the cell count for the 8 controls gave 1743 dividing cells to 13,501 cells counted, i.e., 12·8 per cent.

This laborious work with the control seedlings revealed some interesting facts which had to be taken into consideration in the further investigations of the irradiated rye plants: (1) the constancy of the number of cells in certain layers — 61–84 cells in the epidermis, 30–40 in the sub-epidermal layer, 4–5 layers of cells in the periblem with a number of cells varying between 20 and 35, while in the first layer of the central

cylinder the cell count rose sharply reaching 47 and again fell off sharply from the second layer of this tissue. There was usually one large cell, rarely two, in the centre of the root, the constancy of the number of sub-epidermal cells being particularly striking; and (2) the cell structure of the tissue of rye root is characterized by cylindrical cells in the epidermis, becoming elongated in the subsequent layers and cylindrical again in the central cylinder.

TABLE 3. CONTROL PLANT NO. 1

| Cell layer | Total number of cells in section | | | | | |
|---|---|---|---|---|---|---|
| | First | | Second | Third | Fourth | Fifth |
| Epidermis | 1 | 70 | 66 | 66 | 62 | 67 |
| Sub-epidermal layer | 2 | 35 | 34 | 33 | 32 | 33 |
| Subsequent layers | 3 | 25 | 29 | 27 | 23 | 27 |
| Periblem | 4 | 24 | 21 | 24 | 21 | 28 |
| | 5 | 25 | 19 | 20 | 19 | 22 |
| | 6 | 24 | 18 | 19 | 26 | 29 |
| Central cylinder | 7 | 44 | 22 | 38 | 41 | 43 |
| Central axial cylinder layers in section | 8 | 35 | 31 | 33 | 26 | 35 |
| | 9 | 21 | 26 | 17 | 16 | 19 |
| | 10 | 15 | 15 | 16 | 15 | 18 |
| Central cell | 11 | 1 | 1 | 1 | 1 | 1 |
| Total cell count | 315 | | 282 | 300 | 282 | 322 |
| Number of dividing cells | 45 | | 44 | 37 | 37 | 37 |
| Percentage of dividing cells | 14·3 | | 15·5 | 12·3 | 13·1 | 12·7 |

TABLE 4. NUMBER OF DIVIDING CELLS IN IRRADIATED SEEDLINGS (PLANT NO. 2)
(γ-rays, dose 341 r over 27 days)

| Cell layer* | Total number of cells in each section | | | | |
|---|---|---|---|---|---|
| | First | Second | Third | Fourth | Fifth |
| 1 | 74 | 81 | 79 | 80 | 84 |
| 2 | 38 | 40 | 37 | 36 | 38 |
| 3 | 27 | 26 | 26 | 28 | 26 |
| 4 | 23 | 21 | 23 | 25 | 22 |
| 5 | 26 | 20 | 23 | 21 | 23 |
| 6 | 24 | 22 | 23 | 26 | 21 |
| 7 | 24 | 28 | 25 | 39 | 26 |
| 8 | 36 | 35 | 42 | 34 | 38 |
| 9 | 32 | 36 | 33 | 22 | 22 |
| 10 | 20 | 20 | 20 | 16 | 15 |
| 11 | 15 | 12 | 16 | 1 | 1 |
| 12 | 1 | 1 | 1 | — | — |
| Total cell count | 340 | 342 | 348 | 328 | 316 |
| Number of dividing cells | 61 | 55 | 53 | 60 | 51 |
| Percentage of dividing cells | 17·9 | 16·0 | 15·2 | 18·2 | 16·1 |

* Cell layers numbered as in Table 3.

We shall now consider the behaviour of the cells in the irradiated seedlings taking as our example the root sections of the second plant (Table 4), with a summary of the complete results obtained for the seven irradiated plants. The most conspicuous feature in comparing the two tables is the general increase in the number of dividing cells; on average for the irradiated roots for a total cell count of 9125 in five sections there were 1704 dividing cells, or 18·6 per cent.

Table 5 compares the number of dividing cells in the control plants and those treated by chronic irradiation.

TABLE 5. NUMBER OF RESTING AND DIVIDING CELLS (IN FIVE SECTIONS)

| Experimental variant | Total cell count | Number of dividing cells | % of dividing cells |
|---|---|---|---|
| Controls comprizing 8 plants | 13,501 | 1743 | 12·8 |
| Six irradiated plants (γ-rays, dose 341 r over 27 days) | 9,125 | 1704 | 18·6 |

There was thus a 5·8 per cent increase in the number of dividing cells in the irradiated plants and much the same figure was obtained on more accurate calculation of the number of divisions. In order to make the data more accurate, the average numbers of cells per section and of dividing cells per section and the percentage number of dividing cells were obtained and subjected to statistical treatment (Table 6).

TABLE 6

| Experimental variant | Average number cells per section | Average number of dividing cells per section | % of dividing cells per section |
|---|---|---|---|
| Controls | 300±2·4 | 38·7±2·6 | 12·9 |
| Irradiated plants (γ-rays, dose 341 r over 27 days) | 304·1±2·5 | 56·8±1·2 | 18·6 |

As these tables show, by using large numbers and γ-irradiation, the same pattern was observed as with X-rays, with stimulation of growth and development and thereby a raised yield of the plants, in this case rye, with an increased number of dividing cells in the initial phase of development. It would, therefore, be extremely interesting to compare the action of other stimulatory agents on cell behaviour in these and other plants.

TABLE 7. CHANGE IN NUMBER OF DIVIDING CELLS AFTER IRRADIATION (γ-RAYS) IN SUB-EPIDERMAL LAYER

| Variants | Total cell count | Number of dividing cells | Percentage of dividing cells |
|---|---|---|---|
| Controls | 15,160 | 1937 | 12·77 |
| Action of γ-rays (341 r over 27 days) | 4,992 | 846 | 16·94 |

We were also interested in the methodological aspects of our cytological examinations. Until now in determining the number of dividing cells, we have calculated the divisions in only one layer of the sub-epidermis, and therefore in these preparations we counted the total number of cells in the sub-epidermal layers and the number of dividing cells among them. As Table 7 shows the difference between the results of these and the other counts was not so large as to necessitate the laborious work entailed in counting all the cells in one section.

All our cytological investigations were made with fixed cells stained with haematoxylin. Although this method requires a longer time to achieve results, we were able to investigate the changes induced by irradiation not only in the nucleus and chromosomes but over the whole cell with all its functional parts. As a result we were able to detect changes in the hyaloplasm of the irradiated cells. We shall devote a special article to this problem. All the cell counts both on the unirradiated and irradiated specimens were made by one person in order to avoid different criteria in determining the phases of division. The sections were prepared by V. A. Iazykova and all the calculations carried out by Z. F. Mileshko to whom I express my thanks.

VOL
5
196

## DISCUSSION

Much research has been devoted to the effects of ionizing radiations but those of the greatest interest to us were the investigations in which the time factor was considered as, for example, the work of Trudova and Shekhtman [1] in which the mitotic, activity in irradiated wheat grains was studied.

The data obtained agree with the results of the work by Shekhtman in which he showed that the "time factor" in irradiation of wheat seedlings is manifest only when the duration of irradiation exceeds 4–6 hr. Also of interest are the data obtained by Luchnik [2] on the influence of fractionation and strength of dose on the cytological effect of irradiation in which attention was also paid to the time factor.

The assumption made by us in 1935 on the relationship between the rapidity of cell division, the stimulation of development, and the increased yield on the basis of cytological and physiological investigations on rye [3] has been confirmed on the pea [4]. It was not confirmed however in investigations on wheat in which no change was noted in the speed of cell division and a stimulatory effect was absent [5].

Our assumption has been developed into a hypothesis by the investigations of our colleagues in the Urals Branch of the U.S.S.R. Academy of Sciences, headed by Timofeyev-Resovskii [6–8], who on extensive material covering several species of plants have been able to confirm it. These findings are not only of theoretical interest, but of great practical importance since they mean that the doses of irradiation which can increase the harvest may be fixed without the expensive and immensely laborious investigations into germination and sprouting. Until the joint efforts of cytologists, physiologists and biochemists are able to establish the mechanism of action of radiations, we can only approach the problem of the stimulatory effect empirically. The favourable cytological changes detected by us, including the speed of cell division, enable the

cytologist in the winter season during preparations for sowing, to define the amount of radiation necessary to increase not only the general bulk of the crop but also the content of substances present in plants of industrial and medicinal importance.

## SUMMARY

(1) The action of ionizing radiations in small doses over a lengthy period of time has been investigated.

(2) The method of irradiating fruits and seeds of various plants for 27 days before sowing has shown that with prolonged irradiation the dose of 1000 r for X-rays can be cut to one-third with an increased yield of 20 per cent.

(3) Prolonged γ-irradiation at a dose of 341 r for 27 days accelerated cell division in the root meristem by 6 per cent as compared with cell division in unirradiated cells.

(4) The investigation has confirmed a relationship between the acceleration of cell division and the stimulatory effect.

*Translated by* A. CROZY

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* There is a translation into English of this work.

VOL. 5 1960

# THE RADIOSENSITIVITY AND EMBRYOGENESIS OF *ASCARIS SUIS* OVA*

I. D. VINOGRADOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
5
196

IT is well known that the radiosensitivity of individual organs and tissues is correlated with their mitotic activity. Consequently changes in radiosensitivity during mitosis and embryonic development are of considerable scientific interest, since they provide an indication of the fundamental mechanisms of radiation damage. In addition, study of radiosensitivity during embryogenesis is of considerable practical value in the elucidation of many questions in radiation genetics and radiation protection studies.

Changes in radiosensitivity during the division of egg cells were first observed a comparatively long time ago [1, 2] and subsequently they were studied in detail by several authors [3–8]. These workers studied radiosensitivity mainly during the early stages of development.

The aim of the present work was to study radiosensitivity in ascarid ova from the single blastomere stage to the mature embryo.

Ova obtained from *Ascaris suis* were used for this study of the relation between radiosensitivity and stage of development. This material was chosen because the developmental cycle of *A. suis* ova from fertilization to motile invasive larva is relatively long drawn-out. The blastula stage is reached in 22 hr in amphibia and in 9–10 hours in the sea urchin, whereas in *A. suis* it is reached only after 180 hr. The time which elapses before the first division is mainly responsible for the irereased duration of the developmental cycle of *A. suis*. In *A. suis* the first division cleft appears 40–50 hr after discharge of the ova from the uterus, whereas the corresponding period in amphibia is 4 hr and in sea urchins 1 hr. The length of the developmental cycle in the case of *A. suis* allows radiosensitivity changes to be studied in greater detail, especially during the early stages of development. There are disadvantages in the use of this material, it is true, since the time of fertilization of the ova cannot be determined accurately, thus making precise estimates of the age of embryos difficult. But, since ascarid ova are strictly aerobic, their development commences only after they have been released from the uterus into the external medium. In view of this, the time of discharge of the ova from the uterus was taken as the beginning of development and the age of the embryo calculated from this point.

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Specimens of *A. suis* were obtained from the slaughter-house within 2 hr of removal from the host animal. They were dissected in the laboratory and fertilized eggs were removed from the terminal segment of the uterus 7–10 cm from the genital pore. Eggs taken from different individuals had slightly different radiosensitivities (Fig. 1).

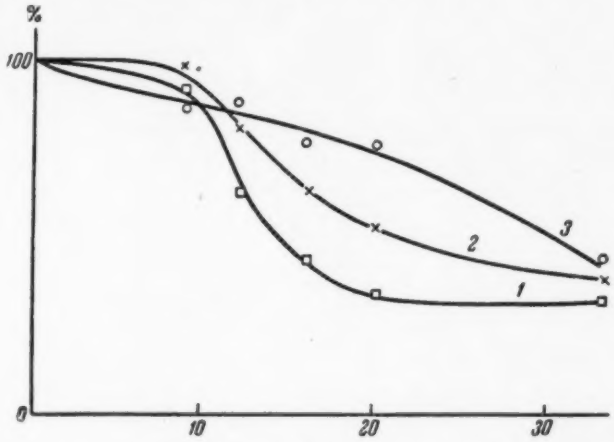


FIG. 1. The radiosensitivity of batches of *Ascaris suis* ova obtained from different worms. The average number of blastomeres per sample on the sixth day of development (as percentages of the control value) is given along the ordinate, and the radiation dose (in krad) along the abscissa; curves *1*, *2* and *3* represent different worms.

Therefore a mixed culture of ova was used in the experiments; ova were taken from corresponding sectors of the uterus of several individuals in order to obtain as uniform a culture as possible. The adhering uterine contents were removed by repeated centrifugation in a 5% solution of NaCl. The washed and centrifuged suspension was divided into a number of samples corresponding to the number of tests to be carried out. Plastic dishes, containing a solution consisting of one part formal-saline (3% formalin in physiological saline) to two parts water, were used for irradiation and subsequent cultivation of the ova.

A beryllium window X-ray tube (type BF 70) was used as the radiation source (60 kV, 40 mA, 0·25 Al filter); the effective wave length was around 0·7 Å. The dose rate at a distance of 10 cm from the window was 4,000 rad/min.

A dose of 20,000–30,000 rad produced a 50 per cent inhibition in the development of *A. suis* ova. The dose curve was sigmoid in form (Fig. 2). From Fig. 2 it can be seen that there was no difference in the number of surviving embryos on the 40th and 17th days of the experiment. Consequently it was concluded that embryos damaged by irradiation died within a period of 17 days.

When eggs at the single blastomere stage were irradiated immediately after they had been taken from the uterus, death occured at different stages of development,

depending on the dose (Table 1). It can be seen from Table 1 that there was a considerable inhibition of the development of irradiated ova in comparison with the control, and that a dose of 100,000–200,000 rad caused an inhibition of the development of ova at the very early stages (2–16 blastomeres).

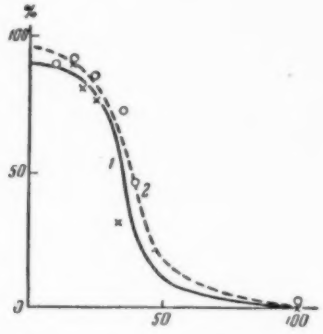


FIG. 2. The relation of the dose of irradiation to survival of ova. The number of mature larvae is given along the ordinate as the percentage of the total number of ova per sample left on the 40th day — curve *1*, and on the 17th day after irradiation — curve *2*. The radiation dose is given along the abscissa (krad). The ova were irradiated immediately after removal from the uterus.

An experimental investigation of changes in radiosensitivity during embryogenesis was carried out as follows. After cleaning and mixing, the eggs to be used in the experiment were divided into two batches. One batch was used immediately. The

TABLE 1. THE INHIBITION OF DEVELOPMENT OF *Ascaris suis* OVA FOLLOWING IRRADIATION

| Dose (Krad) | Number of larvae at a given stage of development 17 days after irradiation (as percentages of the total number of embryos in the sample) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Dead | 1 blast. | 2 blast. | 4 blast. | 8 blast. | 16 blast. | Morula | Blastula | Gastrula | Motile larva |
| Control | 2 | — | 0·5 | — | — | — | — | — | — | 97·5 |
| 10 | 1·5 | — | 0·5 | — | — | — | 0·5 | 6 | — | 91·5 |
| 16 | — | — | — | — | — | — | — | 6 | — | 94 |
| 20 | 2 | — | — | — | — | — | — | 5 | — | 93 |
| 25 | 1 | — | — | — | — | — | 1 | 10·5 | — | 87·5 |
| 34 | — | — | 0·5 | — | — | — | 3 | 23 | — | 73·5 |
| 40 | 0·5 | — | — | — | — | — | 7 | 44 | 1 | 46·5 |
| 100 | 1·5 | — | 2·5 | 11·5 | 27·5 | 57·5 | — | — | — | — |
| 200 | 2 | — | 25 | 25 | 41 | 7 | — | — | — | — |

other batch was stored in a refrigerator at +4°C. for 17 hr in order to delay development of the ova and to shift the time of irradiation from night to day. It was established by separate experiments that even prolonged storage of ova at +4°C. had no appreciable

effect on their viability (Table 2). Besides this, a number of samples taken from the first and second batches were irradiated at the same stage of development to exclude any possible effect of cooling. The results obtained in these control experiments with eggs of the first and second batches corresponded exactly to those of the experiment as a whole.

TABLE 2. THE EFFECT OF COOLING ON THE DEVELOPMENT OF *Ascaris suis* OVA (AVERAGE NUMBER OF BLASTOMERES PER OVUM ON THE SIXTH DAY OF DEVELOPMENT AT +25·5°C.)

| Duration of cooling | Temperature of cooling (°C) | |
|---|---|---|
| | +5 | −10 |
| Control (not cooled) | 3·7 | |
| 7 days | 4·0 | 3·9 |
| 14 days | 4·0 | 3·8 |
| 54 days | 3·0 | 3·3 |

VOL. 5 1960

Consequently it was possible to run the experiment for a period of 400 hours with 4 hour intervals between observations. Each sample was examined prior to irradiation to determine the stage of development of the ova. The ova were cultured at +25·5°C. before and after irradiation. The number of ova, which reached the invasive larva stage 31–32 days after the commencement of development, was taken as the criterion of radiosensitivity.

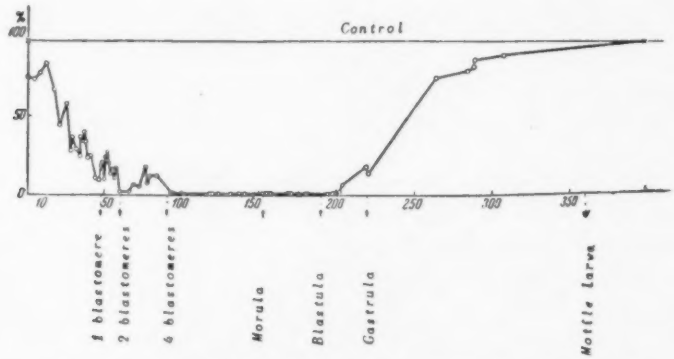


FIG. 3. Radiosensitivity changes during the developmental cycle of ova from *Ascaris suis* (23,500 rad)· The number of live larvae on the 17th day after irradiation of the last sample (as percentages of the total number of larvae in each sample) is given along the ordinate. The stage of development when the ova were irradiated, in hours, is given along the abscissa; the arrows indicate division times.

During the first part of the investigation all the ova received a dose of 23,500 rad. The results are shown in Fig. 3. It can be seen from this curve that there was a ten-fold change in radio-sensitivity during embryogenesis. Whereas the ova were only very slightly sensitive to irradiation during the first 4–10 hr of development (70 per

cent survival), there was a marked increase in the percentage of embryos dying by the time the first division cleft appears. Only 10 per cent of embryos survived to the invasive larva stage in the samples which had been irradiated at the 48-hr stage of development. The 23,500 rad dose resulted in 100 per cent mortality among embryos from the 90–100-hr stage (8–16 blastomeres) up to the late blastula stage. There was a gradual increase in the resistance of embryos from the late blastula stage to the invasive larva stage; at the 408-hr stage of development the percentage of surviving larvae reached the control value (96·5 per cent).

Since the 23,500 rad dose destroyed all ova from the 8–16 blastomere stage up to the early gastrula stage, changes in radiosensitivity during this period could not be detected.

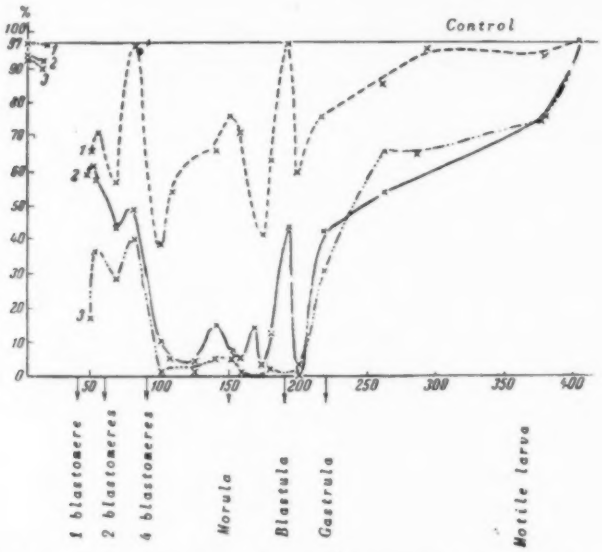


FIG. 4. Radiosensitivity changes during the developmental cycle of ova from *Ascaris suis* (5000, 10,000 and 16,000 rad doses). The number of live larvae on the 17th day after irradiation of the last sample (as percentages of the total number of ova in each sample) is given along the ordinate. The stage of development when the ova were irradiated, in hours, is given along the abscissa. Curve *1* — 5000 rad, curve *2* — 10,000 rad, curve *3* — 16,000 rad.

Lower dosages (5000, 10,000 and 16,000 rad) were employed, therefore, to study radiosensitivity between the fourth division stage and the early gastrula stage.

As in the first series of experiments, the ova used were from the same batch of material and they were irradiated and cultured under standard conditions. The results are shown in Fig. 4. It can be seen that there was a regular change in radiosensitivity during development both at early stages of division (divisions I and II) and between the third division and the early gastrula stage. There was a close correspondence be-

tween the curves for different doses, and radiation damage, i.e. the number of eggs destroyed increased with increasing dose.

In contrast to Fig. 3, the radiosensitivity curves in Fig. 4 exhibit a series of new periodic variations in radiosensitivity: (1) a decline in radiosensitivity between the 8 blastomere (fourth division) and morula stages; (2) an increase in radiosensitivity between the morula and blastula stages; (3) a second decline in radiosensitivity in the middle of the blastula stage and an increase in radiosensitivity during the early gastrula stage; and finally (4) a gradulal decline in radiosensitivity from the early gastrula stage up to the invasive larva stage.

If our results for the radiosensitivity of ascarid ova at the first division stage (Fig. 3) are compared with similar data for the ova of frogs (Shekhtman [5]) and leach (Neifakh [7]), it will be noted that there was only one radiosensitivity maximum during the period up to the end of the first division, whereas in the work on frog and leach ova an additional earlier increase in radiosensitivity was observed, which coincided with the end of the first reduction division. This difference is evidently a result of the fact that in the case of ascarid ova fertilization and the reduction division occur within the body of the parent worm and only the first somatic nuclear division can be observed in this material.

VOL. 5 1960

The author wishes to thank Prof. Ia. L. Shekhtman for his guidance and assistance in this work and for helpful discussion of the results.

## SUMMARY

Periodic variations have been observed in the radiosensitivity of developing ascarid ova, which were obviously connected at early stages of division (the first three divisions) with the process of mitosis, and at later stages with damage to sectors of tissue and organs of vital importance for the further development of the organism as a whole.

*Translated by* C. PRINGLE

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# THE α-RAY DOSE RATE NEAR AN INTERFACE BETWEEN DIFFERENT MEDIA*

G. A. VOLKOV and G. R. RIK

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IT is often necessary to calculate α-ray dose rates in radiotherapy, applied physics, and so on. It is especially difficult to calculate the α-ray dose rate near the interface between different media, which may differ in stopping power, in isotope content, and in nature of isotope. There are three main types of interface, namely flat, cylindrical, and spherical. Formulas relating to flat surfaces have been used with various approximations [1]. The approximations are often justified, because the corrections are small, but in exact work, or in work with small objects, the approximations are too rough.

Spherical surfaces have been considered recently [2]; so have cylindrical surfaces [3]. But in all cases it has been asumed that the two media contain the same emitter, and (more important) that the energy lost per unit path is constant. The treatment given here is free from these restrictions; we assume that the energy lost between the distances to a point $r$ and to the end of the range $R$ is governed by the reasonably exact empirical law [4]

$$E = K_0 \varepsilon [R - r]^{2/3}, \tag{1}$$

in which $\varepsilon$ is the energy required to produce one ion-pair, and $K_0$ is a constant $6{\cdot}253 \times 10^4$. Then (1) gives us directly that the initial energy is

$$E_\alpha = K_0 \varepsilon R^{2/3}. \tag{2}$$

The energy absorbed in unit time in a volume $V$ within a homogeneous medium whose dimensions greatly exceed $R$ is

$$E_0 = NE_\alpha V = NK_0 \varepsilon R^{2/3} V, \tag{3}$$

where $N$ is the number of α rays originating in unit time in unit volume.

Now if near $V$ we have a medium that differs in properties from the first, then the energy as defined in (3) is given by

$$P = \frac{E_0}{V} + \frac{\Delta E}{V}, \tag{4}$$

in which $\Delta E$ allows for the difference between the energy lost from $V$ and the energy entering V. If the two media are identical

$$\Delta E = 0. \tag{5}$$

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Suppose now we have two different media I and II (Fig. 1), whose parameters are $N_j$ (analogous to $N$), $E_{\alpha j}$ (analogous to $E_\alpha$), $R_j$ (the range for $\alpha$ rays of energy $E_{\alpha j}$ in medium $j$), $R_j'$ (the range of the $\alpha$ rays from the adjacent medium), $V_j$ (the volume

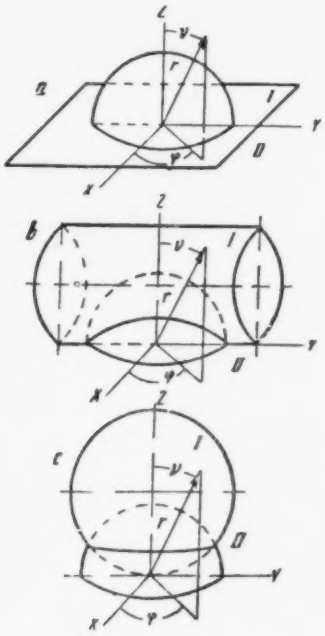

FIG. 1.

within $j$ from which $\alpha$ rays enter the adjacent medium), $V_j'$ (the volume within $j$ which is accessible to $\alpha$ rays from the adjacent medium), $b$ is a parameter specifying the size of the medium (diameter of sphere or cylinder), and

$$k = \frac{R_1}{b} \tag{6}$$

We shall consider the most important case, namely $k < 1$.

(1) *Flat interface.* The energy absorbed by II from medium I (Fig. 1, a) in unit time through unit surface $\sigma$ is given by

$$\frac{dE_1}{d\sigma} = \frac{N_1 K_0 \varepsilon}{\pi} \int_0^{\pi/2} d\varphi \int_0^{\pi/2} \cos\vartheta \sin\vartheta d\vartheta \int_0^{R_1} (R_1 - r)^{2/3} dr . \tag{7}$$

Integration and use of (2) gives

$$\frac{dE_1}{d\sigma} = \frac{3}{20} N_1 E_{\alpha 1} R_1 . \tag{8}$$

The energy flux is found by integrating (8); it is

$$E_1 = \frac{3}{20} N_1 E_{\alpha 1} R_1 \sigma . \tag{9}$$

Then (5) and (9) give

$$E_1 \Big|_{\substack{N_1 = N \\ NR_1 = R \\ E_{\alpha 2} = E_\alpha}} = E_2 \Big|_{\substack{N_2 = N \\ R_2 = R \\ E_{\alpha 2} = E_\alpha}} = \frac{3}{20} N E_\alpha R \sigma \tag{10}$$

or

$$E_2 = \frac{3}{20} N_2 E_{\alpha 2} R_2 \sigma . \tag{11}$$

The volumes accessible to one medium from the other ($E_{\alpha 1} > E_{\alpha 2}$) are

$$V_1 = \sigma R_1 \tag{12}$$

$$V_2' = \sigma R_2', \tag{13}$$

and (3), (4), (12) and (13) give the mean dose rate as

$$P_1 = E_{\alpha 1} N_1 \left\{ 1 - \frac{3}{20} \left[ 1 - \frac{N_2 E_{\alpha 2} R_2}{N_1 E_{\alpha 1} R_1} \right] \right\}; \tag{14}$$

$$P_2 = E_{\alpha 2} N_2 \left\{ 1 - \frac{3}{20} \frac{R_2}{R_2'} \left[ 1 - \frac{N_1 E_{\alpha 1} R_1}{N_2 E_{\alpha 2} R_2} \right] \right\} \tag{15}$$

A factor $R_1/R_1'$ must be inserted before the square bracket in (14) if $E_{\alpha 1} < E_{\alpha 2}$; the factor $R_2/R_2'$ in (15) then left out.

(2) *Cylindrical interface.* The energy absorbed by II from medium I (Fig. 1, b) in unit time through unit surface $\sigma$ is given by

$$\frac{dE_1}{d\sigma} = \frac{dE_{11}}{d\sigma} + \frac{dE_{12}}{d\sigma}, \tag{16}$$

$$\frac{dE_{11}}{d\sigma} = \frac{N_1 K_0 \varepsilon}{\pi} \int_0^{\pi/2} \int_0^{\alpha'(\varphi)} \cos\vartheta \sin\vartheta \, d\vartheta \, d\varphi \int_0^{R_1} (R_1 - r)^{2\,3} \, dr \tag{17}$$

in which

$$\frac{dE_{12}}{d\sigma} = \frac{N_1 K_0 \varepsilon}{\pi} \int_0^{\pi/2} \int_0^{\pi/2} \cos\vartheta \sin\vartheta \int_0^{\varrho(\vartheta,\varphi)} (R_1 - r)^{2/3} \, dr . \tag{18}$$

Here $\alpha'(\varphi)$ is the $\vartheta$ that satisfies the equation for the line in which the cylinder and sphere of radius $R_1$ meet:

$$\cos\vartheta = k(\cos^2\varphi + \sin^2\varphi \cos^2\vartheta), \tag{19}$$

VOL 5 196

and $\rho(\vartheta, \varphi)$ is the distance from the origin to a point on the cylinder:

$$\rho(\vartheta, \varphi) = b \cos \vartheta \frac{1}{1 - \sin^2\varphi \sin^2\vartheta}. \tag{20}$$

Integrating (17) for $r$ and $\vartheta$ gives

$$\frac{dE_{11}}{d\sigma} = \frac{N_1 E_{\alpha 1} R_1}{\pi} \frac{3}{10} \int_0^{\pi/2} \sin^2\alpha'(\varphi)\, d\varphi. \tag{21}$$

Calculations [3] show that the integral in (21) takes the form

$$\int_0^{\pi/2} \sin^2\alpha'(\varphi)\, d\varphi = \frac{2}{3}[k + D - 2k^2 D], \tag{22}$$

in which $K$ and $D$ denote the corresponding complete elliptic integrals. The (17), (21), and (22) give

$$\frac{dE_{11}}{d\sigma} = \frac{N_1 E_{\alpha 1} R_1}{\pi} \frac{1}{5}[K + D - 2k^2 D]; \tag{23}$$

Now $K$ and $D$ may be represented as power series in $k$:

$$K = \frac{\pi}{2}\left\{1 + \sum_{n=1}^{\infty}\left[\frac{(2n-1)!!}{2^n n!}\right]^2 k^{2n}.\right. \tag{24}$$

$$D = \pi \sum_{n=1}^{\infty} \frac{n}{(2n-1)}\left[\frac{(2n-1)!!}{2^n n!}\right]^2 k^{2(n-1)} \tag{25}$$

We may use only such terms as are appropriate to the accuracy we require; then, for example, to powers no higher than $k^2$, we have for (23) the expression

$$\frac{dE_{11}}{d\sigma} = \frac{3}{20} N_1 E_{\alpha 1} R_1 [1 - 0{\cdot}3750 \cdot k^2]. \tag{26}$$

We integrate (18) with respect to $r$ and $\vartheta$ and get

$$\frac{dE_{21}}{d\sigma} = \frac{N_1 E_{\alpha 1} R_1}{\pi} \frac{3}{5} \sum_{q=1}^{\infty} (-1)^{q+1} \binom{5/3}{q} \sum_{p=0}^{\infty} \frac{(q+p-1)!}{(q-1)!\, p!} \cdot$$

$$\cdot \sum_{l=0}^{p} (-1)^l \binom{p}{l} \frac{1}{q+2l+2} k^{-q} \int_0^{\pi/2} \sin^2\varphi \cos^{q+2l+2}\alpha'(\varphi)\, d\varphi. \tag{27}$$

Now $\cos x\alpha'(\varphi)$ may be expressed as a power series in $k$ [3]:

$$\cos^x \alpha'(\varphi) = \sum_{n=x}^{\infty} (-1)^n 2^{2n-x} A_{x,n} k^{2n-x} \cos^{2n}\varphi \sin^{2n-2x}\varphi, \tag{28}$$

in which

$$A^{x,n} = \sum_{j=1}^{x} (-1)^j \binom{x}{j} a_{j,n}, \tag{29}$$

$$a^{j,n} = \frac{1}{n} \sum_{i=1}^{n} \binom{1/2}{i} [(j+1)i - n] a_{j,n-1}, \tag{30}$$

$$a_{j,0} = 1 \tag{31}$$

We integrate (27) with respect to $\varphi$ and use (28)–(31) to get

$$\frac{dE_{12}}{d\sigma} = \frac{N_1 E_{\alpha 1} R_1}{\pi} Q,$$

$$Q = \frac{3}{5} \sum_{q=1}^{\infty} (-1)^{q+1} \binom{5/3}{q} \sum_{p=0}^{\infty} \frac{(q+p-1)!}{(q-1)!\,p!} \sum_{l=0}^{p} (-1)^l \binom{p}{l} \frac{1}{q+2l+2}. \tag{32}$$

in which

$$\sum_{n=q+2l+2}^{\infty} (-1)^n 2^{2n-2l-q-2} A_{2l+q+2,n} k^{2n-2l-2q-2} \sum_{m=0}^{p+n-2l-q-2} (-1)^m \cdot$$

$$\cdot \binom{p+n-2l-q-2}{m} \frac{[(2n+2m)-1]!!}{2^{n+m}(m+n)!} \cdot \frac{\pi}{2}. \tag{33}$$

We take only the terms up to $k^2$ in (33); that is, we put $l = 0$, $n = q+2$, and get

$$Q\Big|_{\substack{l=0 \\ n=q+2}} = \frac{3}{5} \sum_{q=1}^{\infty} (-1)^{q+1} \binom{5/3}{q} \sum_{p=0}^{\infty} \frac{(q+p-1)!}{(q-1)!p!} \frac{1}{(q+2)} k^2 \cdot$$

$$\cdot \sum_{m+0}^{p} (-1)^m \binom{p}{m} \frac{[(2m+2q+4)-1]!!}{2q+^{m+2}(q+m+2)!} \cdot \frac{\pi}{2}. \tag{34}$$

Values of $Q$ given by (34) are:

| | | | | |
|---|---|---|---|---|
| 0 | 0·0527 | 0·0406 | 0·0397 | 0·0395 |
| 1 | 0·0586 | 0·0449 | 0·0438 | 0·0435 |
| 2 | 0·0606 | 0·0461 | 0·0449 | 0·0446 |
| 3 | 0·0614 | 0·0465 | 0·0452 | 0·0449 |
| 4 | 0·0618 | 0·0467 | 0·0454 | 0·0451 |
| 5 | 0·0621 | 0·0469 | 0·0458 | 0·0453 |

These values of $Q$ are such that the difference between $p = 4$ and $p = 5$ is only 0·3 to 0·5 per cent, which is a permissible error. The difference is about 3 per cent between the $Q$ for $q = 3$ and $q = 5$ ($p = 5$), and for $q = 3$ and $q = 4$ ($p = 5$) it is about 0·7 per cent. Therefore it is sufficient to stop at this point and use the value

$$Q = 0{\cdot}0453\,\pi k^2. \tag{35}$$

Larger values of $q$ must be used if $Q$ must be known more exactly.

Now (32), with (35), becomes

$$\frac{dE_{12}}{d\sigma} = \frac{3}{20} N_1 E_{\alpha 1} R_1 \cdot 0{\cdot}3020 k^2 . \tag{36}$$

and, using (23) and (32), we get (16) as

$$\frac{dE_1}{d\sigma} = \frac{N_1 E_{\alpha 1} R_1}{\pi} \frac{1}{5} [K+D+5Q-2k^2 D], \tag{37}$$

or, using (26) and (36), and restricting ourselves to $k^2$, we have

$$\frac{dE_1}{d\sigma} = \frac{3}{20} N_1 E_{\alpha 1} R_1 [1-0{\cdot}0730 \cdot k^2]. \tag{38}$$

VOL. 5 1960

The energy transmitted from I to II in unit time through the surface of a cylinder of height $h$ is found by integrating (37) with respect to $\sigma$ as

$$E_1 = N_1 E_{\alpha 1} R_1 bh \frac{1}{5} [K+D+5Q-2k^2 D]. \tag{39}$$

Then (5) and (39) give us directly that

$$E_1 \Big|_{\substack{N_1=N \\ R_1=R \\ E_{\alpha 1}=E_\alpha}} = E_2 \Big|_{\substack{N_2=N \\ R_2=R \\ E_{\alpha 2}=E_\alpha}} = NE_\alpha Rbh \frac{1}{5} ([K+D+5Q-2k^2 D], \tag{40}$$

or

$$E_2 = N_2 E_{\alpha 2} R^2 bh \frac{1}{5} [K+D+5Q-2k^2 D] \tag{41}$$

The volumes accessible to one medium from the other ($E_{\alpha 1} > E_{\alpha 2}$) are

$$V_1 = \pi h R_1^2 \left[\frac{1}{k} - 1\right], \quad R_1 < \frac{b}{2}; \tag{42}$$

$$V_1 = \pi h R_1^2 \frac{1}{4k^2}, \quad R_1 \geqslant \frac{b}{2}; \tag{43}$$

$$V_2' = \pi h R_2'^2 \left[1 + \frac{R_1}{R_2'} \frac{1}{k}\right]. \tag{44}$$

and (4), (3), (39), (41)–(44) give the mean dose rate as

$$P_1 = N_1 E_{\alpha 1} \left\{1 - \frac{1}{5\pi(1-k)} \left[1 - \frac{N_2 E_{\alpha 2} R_2}{N_1 E_{\alpha 1} R_1}\right] \cdot \right.$$

$$\left. \cdot [K+D+5Q-2k^2 D]\right\}, \quad R_1 < \frac{b}{2}; \tag{45}$$

$$P_1 = N_1 E_{\alpha 1}\left\{1 - \frac{4}{5\pi} k\left[1 - \frac{N_2 E_{\alpha 2} R_2}{N_1 E_{\alpha 1} R_1}\right]\cdot\right.$$

$$\left.\cdot\,[K + D + 5Q - 2k^2 D]\right\},\quad R_1 \geqslant \frac{b}{2}\,; \tag{46}$$

$$P_2 = N_2 E_{\alpha 2}\left\{1 - \frac{1}{5\pi\left(1 + k\frac{R'}{R_1}\right)} \frac{R_2}{R_2'}\left[1 - \frac{N_1 E_{\alpha 1} R_1}{N_2 E_{\alpha 2} R_2}\right]\cdot\right.$$

$$\left.\cdot\,[K + D + 5Q - 2k^2 D]\right\} \tag{47}$$

or, restricting ourselves to $k^2$, and using (24), (25) and (35) we have

$$P_1 = N_1 E_{\alpha 1}\left\{1 - \frac{3}{20}\,\frac{1}{(1-k)}\,[1 - 0{\cdot}0730 \cdot k^2]\cdot\right.$$

$$\left.\cdot\left[1 - \frac{N_2 E_{\alpha 2} R_2}{N_1 E_{\alpha 1} R_1}\right]\right\},\quad R_1 < \frac{b}{2}\,; \tag{48}$$

$$P_1 = N_1 E_{\alpha 1}\left\{1 - \frac{3}{5} k\,[1 - 0{\cdot}0730 \cdot k^2]\cdot\left[1 - \frac{N_2 E_{\alpha 2} R_2}{N_1 E_{\alpha 1} R_1}\right]\right\},$$

$$R_1 \geqslant \frac{b}{2}\,; \tag{49}$$

$$P_2 = N_2 E_{\alpha 2}\left\{1 - \frac{3}{20}\,\frac{1}{\left(1 + k\frac{R_2'}{R_1}\right)}\,\frac{R_2}{R_2'}\,[1 - 0{\cdot}0730 k^2]\left[1 - \frac{N_1 E_{\alpha 1} R_1}{N_2 E_{\alpha 2} R_2}\right]\right\}. \tag{50}$$

A factor $R_1/R_1'$ must be inserted before the square bracket in (45) and (48) if $E_{\alpha 1} < E_{\alpha 2}$, and $k$ in the round brackets must be multiplied by $R_1'/R_1$; the factor $R_2/R_2'$ before the square brackets in (47) and (50) drops out, and $R_2'$ is replaced by $R_2$ within the round brackets.

(3) *Spherical interface.* The energy absorbed by II from medium I (Fig. 1 c) in unit time through unit surface $\sigma$ is given by

$$\frac{dE_1}{d\sigma} = \frac{dE_{11}}{d\sigma} + \frac{dE_{12}}{d\sigma}\,, \tag{51}$$

in which

$$\frac{dE_{11}}{d\sigma} = \frac{K_1 K_0 \varepsilon}{\pi} \int_0^{\pi/2} d\varphi \int_0^{\alpha} \cos\vartheta \sin\vartheta\, d\vartheta \int_0^{R_1} (R_1 - r)^{3/2}\, dr \tag{52}$$

$$\frac{dE_{12}}{d\sigma} = \frac{N_1 K_0 \varepsilon}{\pi} \int_0^{\pi/2} d\varphi \int_{\alpha}^{\pi/2} \cos\vartheta \sin\vartheta \int_0^{\varrho(\vartheta)} (R_1 - r)^{3/2}\, dr\, d\vartheta\,. \tag{53}$$

Here $\alpha$ is the $\vartheta$ that satisfies the equation for the line in which the surface of medium I meets the sphere of radius $R_1$:

$$\cos\alpha = k, \tag{54}$$

and $\rho(\vartheta)$ is the distance from the origin to a point on the spherical surface of medium I:

$$\rho(\vartheta) = b\cos\vartheta. \tag{55}$$

We integrate (52) for $r$ and $\vartheta$, and get

$$\frac{dE_{11}}{d\sigma} = \frac{3}{20} N_1 E_{\alpha 1} R_1 ([1 - k^2]; \tag{56}$$

and (53) integrates to

$$\frac{dE_{12}}{d\sigma} = \frac{3}{20} N_1 E_{\alpha 1} R_1 T k^2, \tag{57}$$

in which

$$T = 2\sum_{q=1}^{\infty} (-1)^{q+1} \binom{5/3}{q} \frac{1}{(q+2)}. \tag{58}$$

To a high degree of accuracy we may put $T = 0{\cdot}7966$; then (57) becomes

$$\frac{dE_{12}}{d\sigma} = \frac{3}{20} N_1 E_{\alpha 1} R_1 k^2 \cdot 0{\cdot}7966, \tag{59}$$

and (51), taken with (56) and (59) becomes

$$\frac{dE_1}{d\sigma} = \frac{3}{20} N_1 E_{\alpha 1} R_1 [1 - 0{\cdot}2034 \cdot k^2]. \tag{60}$$

The energy flux from I to II through the spherical surface of I is found by integrating (60) with respect to $\sigma$ as

$$E_1 = \frac{3}{20} N_1 E_{\alpha 1} R_1 \pi b^2 [1 - 0{\cdot}2034 \cdot k^2]. \tag{61}$$

Then (5) and (61) give us directly that

$$E_1 \Big|_{\substack{N_1=N \\ R_1=R \\ E_{\alpha 1}=E_\alpha}} = E_2 \Big|_{\substack{N_2=N \\ R_2=R \\ E_{\alpha 1}=E_\alpha}} = \frac{3}{20} N E_\alpha R \pi b^2 [1 - 0{\cdot}2034 \cdot k^2] \tag{62}$$

or

$$E_2 = \frac{3}{20} N_2 E_{\alpha 2} R_2 \pi b^2 [1 - 0{\cdot}2034 \cdot k^2] \tag{63}$$

The volumes accessible to one medium from the other ($E_{\alpha 1} > E_{\alpha 2}$) are

$$V_1 = \frac{1}{6}\pi b^3 k\,[6 - 12k + 8k^2],\quad R_1 < \frac{b}{2}; \tag{64}$$

$$V_1 = \frac{1}{6}\pi b^3,\quad R_1 \geqslant \frac{b}{2}; \tag{65}$$

$$V_2' = \frac{1}{6}\pi b^3 k\,\frac{R_2'}{R_1}\left[6 + 12\,\frac{R_2'}{R_1}\,k + 8\,\frac{R_2^{12}}{R_1^2}\,k^2\right]. \tag{66}$$

and (3), (4), (61), (63–(66) give the mean dose rate as

$$P_1 = N_1 E_{\alpha 1}\left\{1 - \frac{9}{10}\,\frac{1}{(6 - 12k + 8k^2)}\,[1 - 0{\cdot}2034 \cdot k^2]\cdot\right.$$

$$\left.\cdot\left[1 - \frac{N_2 E_{\alpha 2} R_2}{N_1 E_{\alpha 1} R_1}\right]\right\},\quad R_1 < \frac{b}{2}; \tag{67}$$

$$P_1 = N_1 E_{\alpha 1}\left\{1 - \frac{9}{10}\,k\,[1 - 0{\cdot}2034 \cdot k^2]\cdot\left[1 - \frac{N_2 E_{\alpha 2} R_2}{N_1 E_{\alpha 2} R_1}\right]\right\}.\quad R_1 \leqslant \frac{b}{2}; \tag{68}$$

$$P_2 = N_2 E_{\alpha 2}\left\{1 - \frac{9}{10}\,\frac{1}{\left(6 + 12\,\frac{R_2'}{R_1}\,k + 8\,\frac{R_2^{12}}{R_1^2}\,k^2\right)}\,\frac{R_2}{R_2'}\,[1 - 0{\cdot}2034 \cdot k^2]\cdot\right.$$

$$\left.\cdot\left[1 - \frac{N_1 E_{\alpha 1} R_1}{N_2 E_{\alpha 2} R_2}\right]\right\}. \tag{69}$$

A factor $R_1/R_1'$ must be inserted before the square bracket in (67) if $E_{\alpha 1} < E_{\alpha 2}$, and $k$ in the round brackets must be multiplied by $R_1'/R_1$ and $k^2$ by $R_1'^2/R_1^2$; the factor $R_2/R_2'$ before the square brackets in (69) drops out, and $R_2'$ is replaced by $R_2$ within the round brackets.

(4) *Comments.*

(a) These general expressions may be simplified in special cases. If $N_1 = N_2$, $E_{\alpha 1} = E_{\alpha 2}$, and $R_1 = R_2$, we have the result for a homogeneous medium, namely

$$P_1 = P_2 = NE_{\alpha}. \tag{70}$$

In the limit $b \to \infty$ ($k \to 0$) the expressions for the cylindrical and spherical interfaces become those for the flat interface.

(b) If $k \geqslant 1$, the formula (16) for the cylindrical interface has three components, which are

$$\frac{dE_{11}}{d\sigma} = \frac{N_1 K_0 \varepsilon}{\pi}\int_0^{\pi/2}\int_0^{\pi/2}\cos\vartheta\sin\vartheta\int_0^{\varrho(\vartheta,\varphi)}(R_1 - q)^{3/2}\,dr d\vartheta d\varphi, \tag{71}$$

$$\frac{dE_{12}}{d\sigma} = \frac{N_1 K_0 \varepsilon}{\pi} \int_{\frac{1}{2}\arcsin\frac{1}{k}}^{\pi/2} \int_{\alpha''(\varphi)}^{\alpha'(\varphi)} \cos\vartheta \sin\vartheta d\vartheta d\varphi \int_0^{R_1} (R_1 - r)^{3/2} dr, \tag{72}$$

$$\frac{dE_{13}}{d\sigma} = -\frac{N_1 K_0 \varepsilon}{\pi} \int_{\frac{1}{2}\arcsin\frac{1}{k}}^{\pi/2} \int_{\alpha''(\varphi)}^{\alpha'(\varphi)} \cos\vartheta \sin\vartheta \int_0^{\rho(\vartheta,\varphi)} (R_1 - r^{3/2} dr d\vartheta d\varphi \tag{73}$$

and $\alpha''(\varphi) \to 0$ as $k \to 1$, in which $\alpha'(\varphi)$ and $\alpha''(\varphi)$ are the roots of (19). These expressions may be calculated by replacing $\cos\alpha'(\varphi)$ and $\cos\alpha''(\varphi)$ by suitable power-series expansions in $1/k$.

It is sufficient to put $\alpha = 0$ in (52) and (53) for a spherical interface.

(c) A similar argument gives an analogous solution for any law of the form $E \sim (1 - r/R)^y$ for the residual energy of the $\alpha$ rays, if $y$ is an integer.

*Translated by* J. E. S. BRADLEY

VOL. 5 1960

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* There is a translation into English of this work.

# METHODS AND INSTRUMENTS

# AN AUTOMATIC SYSTEM FOR DETERMINING THE DIFFUSION COEFFICIENT*

M. M. ZAALISHVILI, F. O. SHRAIBMAN and A. R. EGIAZAROVA

Institute of Physiology, Academy of Sciences of the Georgian S.S.R., Tbilisi

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## INTRODUCTION

IN physical chemistry two basic constants are considered of great importance for the characterization of the protein molecule. These are the molecular weight and the shape of the molecule. In recent years ultracentrifugation has been sucessfully employed for the determination of these values. The determination of the molecular

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weight requires a knowledge of both the sedimentation constant and the diffusion coefficient. However, since the ultracentrifuge is an uncommon apparatus, measurements of the viscosity and diffusion coefficient are often combined for the determination of the molecular weight and shape of the protein molecule.

For the determination of the diffusion coefficient Lamm's optical method has been used in recent years. Owing to the large size of the protein molecules the diffusion process is very slow, and hence the experiment lasts several days, thus causing considerable inconvenience. Bressler, Finogenov and Pavlova [2] have devised an arrangement which enables the measurements to be made automatically. We have designed a similar arrangement, incorporating valves and mains-operated. It is very compact and works smoothly.

In the study of the interaction of an electrolyte or non-electrolyte with a protein, parallel control experiments must be carried out. Hence it is very important to conduct the experiment with two cells and with the same optical system of lenses and objectives. We used a system of mirrors and a rotating shutter which enabled the light to be directed in turn first through one cell and then through the other.

VOL
5
196

## OPTICAL ARRANGEMENT (SCALE METHOD)

Refraction methods are based on the fact that in media where a concentration gradient exists the beam of light is bent towards the greater optical density. When the zone method is employed the gradient of the refractive index is obtained by direct photography of the optical inhomogeneity of the system in which diffusion occurs. Of these methods we should note the slit method developed by Longsworth [3], and the cylindrical lens method, developed by Thovert [4], Svensson [5] and improved by Philpot [6]. The slit method is more accurate, but is not so accurate as the scale method developed by Lamm [1] and Neurath [7]. Indeed, Lamm's method is the most accurate of the refractometric methods but the comparison of the scales obtained by photography and the calculation of the diffusion coefficient $D$ from the experimental data takes a great deal of time.

A diagram of the instrument which we used for determinations by the scale method is shown in Fig. 1. Light from a mercury lamp SVDSh-250 *1* passes through a condenser and light filter *2*. The filter isolates that part of the spectrum in which the solution does not absorb (green filter). Thus, from the condenser (focal length $f = 50$ cm) and filter there emerges a parallel monochromatic beam of light which passes through the scale *3*, the cell *5* placed in the water thermostat *4*, and is focused by a long-focus objective *8* ($f = 150$ cm) on the photographic plate *9*. The optical bench is mounted on a concrete base to protect the apparatus from mechanical vibrations. The uniform scale was etched on glass. We used a 5 cm-long scale with 0·04 cm divisions. The cell, of similar construction to that described in the work of Bressler and Finogenov [2], is fixed in the water thermostat on a special support. The water thermostat *4* is mounted on the optical bench. Sponge rubber is placed between the thermostat and its support to give better protection against mechanical vibrations. A contact thermometer and stirrer are mounted in the thermostat. The motor for the stirrer is mounted on a special

support, separate from the optical bench, so that the vibrations of the motor will not be transmitted to the cell. The temperature of water in the thermostat is kept constant by means of a thermoregulator.

In front of the long-focus objective there is a diaphragm *7*, which is brought into action by the magnetic shutter *11*. The camera *9* has a slit and is provided with a plate-holder, which is designed so as to allow eight exposures to be taken on one plate. The holder and plate are moved for each successive exposure by means of a Warren motor *10*. All the optical units of the apparatus are mounted on an optical bench *12*. The portions between the thermostat *4* and the diaphragm *7* and between the objective and camera are enclosed in tubes *6* and *13*.

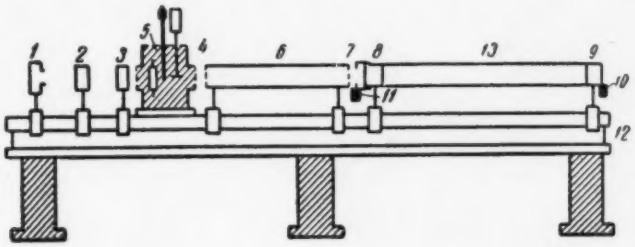


FIG. 1

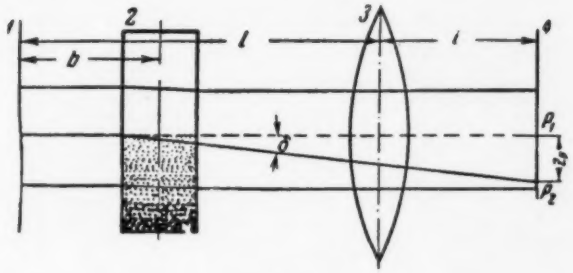


FIG. 2

A thoroughly washed cell is filled with solution and lowered into the thermostat to a point where the interface formed after stratification of the protein solution coincides approximately with the optical axis of the system. When temperature equilibrium is established the protein solution is stratified and the boundary stabilized. The moment of formation of a distinct interface is regarded as the time of onset of diffusion. The scale is focused on the photographic plate and after fixed intervals of time, depending on the diffusion rate, photographs of the scale are made. The exposure must be long enough (more than 5 sec) to avoid the effect of temporary changes of refractive index in the thermostatting liquid. With the plates which we used the exposure was 20 sec.

When diffusion takes place in the cell the concentration varies over the diffusion column and thus a concentration gradient $\frac{\partial c}{\partial x}$ is created. This concentration gradient

produces a gradient of refractive index $\frac{\partial n}{\partial x}$. On passing through a region of the system with a gradient of refractive index the wavefront of the rays, which was vertical before entry into the cell, is gradually bent towards the higher refractive index, whereas rays passing through a region where there is no gradient fall without deflection on the photographic plate (Fig. 2). The angle of deflection of the rays passing through the gradient region is a measure of the gradient of refractive index. Hence, when the light passes through a region of varying refractive index, a displacement of the scale divisions is observed. The amount of the displacement and the position of the displaced lines relative to an arbitrarily chosen line (in the region $x = 0$) also give a measure of the gradient of refractive index.

A comparison of the scales and determination of the positions of the displaced lines (relative to the arbitrarily chosen line) is carried out on the micro-comparator IZA-2 or the universal measuring microscope UIM-21 to an accuracy of one micron.

If the medium in the cell is homogeneous then the beam of light passing through the cell is not deflected. It strikes the photographic plate at point $P_1$ (Fig. 2). If there is a gradient of refractive index in the cell, then the ray is deflected and strikes the plate at point $P_2$. From the theory used by Wiener [8] and Lamm [1] it follows that

$$P_2 - P_1 = Z = Gb\delta, \tag{1}$$

where $Z$ is the displacement of the lines, $G$ the photographic enlargement equal to $i/l$, $\delta$ the angle of deflection of the beam.

Lamm showed that for small deviations the displacement of the beam ($\delta$) was proportional to the cell thickness and the gradient of refractive index $\frac{\partial n}{\partial x}$ at the point where the light passed through the cell, hence

$$\delta = a \cdot \frac{\partial n}{\partial x}. \tag{2}$$

From (1) and (2) we obtain

$$Z_P = G \cdot ab \cdot \frac{\partial n}{\partial x}. \tag{3}$$

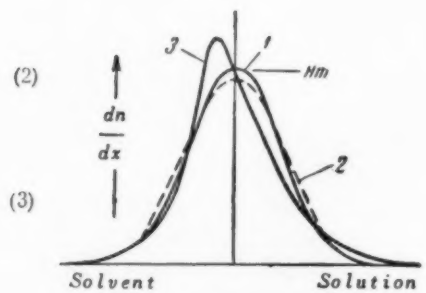


FIG. 3

Thus, by determining the experimental value $Z$, we can calculate $\frac{\partial n}{\partial x}$. In the scale method the photographic enlargement is approximately unity. The dependence of the distance $Z_p$ on the plate (i.e. the distance between point $x = 0$ and $x_p$) on the distance $x_c$ in the cell, according to Fig. 2, can be expressed by the relationship

$$Z = G \cdot \frac{l}{l - b} \cdot x_c . \tag{4}$$

Hence to determine the distance $x$ along the diffusion cell from the displacement $Z$ of the scale division, the values of $Z$ must be multiplied by a proportionality factor $F$ (the geometrical magnification).

$$F = l - b/l \cdot G \tag{5}$$

Values are chosen so that $F$ is close to unity.

The value $Z$ is found as the difference between the position of the scale lines in a control experiment with a solvent ($n_2$) and the position when the protein solution ($n_1$) in the cell is stratified and diffusion is taking place.

## METHOD OF CALCULATING DIFFUSION COEFFICIENT $D$

The calculation of $D$ is based on the relationship:

$$\frac{\partial n}{\partial x} = \frac{n_2 - n_1}{\sqrt{4\pi Dt}} \exp^{(-x^2/4Dt)}, \tag{6}$$

where $n_2$ and $n_1$ are the refractive indices. There are several methods of evaluating equation (6) on the basis of the Gaussian curve. Of these we will discuss the method of areas and maximum ordinate and the statistical method, since these are the methods we use.

(1) *Method of areas and maximum ordinate.* At point $x = 0$ the ordinate has a maximum value ($H = H_m$) and hence equation (6) becomes:

$$\left(\frac{\partial n}{\partial x}\right)^2_{x=0} = H_m^2 \frac{(n_2 - n_1)^2}{4\pi Dt}; \tag{7}$$

whence

$$D = \frac{(n_2 - n_1)^2}{4\pi t H_m^2}. \tag{8}$$

According to (6), however, $n_2 - n_1$ can be replaced by the area $A$ under the curve and if $F$ is not equal to unity (i.e. $Z \neq x$):

$$D = \frac{A^2}{4\pi t H_m^2} \left(\frac{l - b}{lG}\right)^2. \tag{9}$$

Thus, by determining the area under the curve (graphic integration or planimetry) and the value $H_m$, we can calculate $D$.

(2) *Statistical method.* In this method the experimental curves relating $x$ and $\frac{\partial n}{\partial x}$ are regarded as normal distribution curves. According to statistical theory, the Gaussian curve has the form:

$$S = \frac{N\omega}{\sigma\sqrt{2\pi}} \exp^{(-(s-\beta)^2\omega^2/2\sigma)} \tag{10}$$

where $S$ is the statistical frequency, sis the variable, $\beta$ its arithmetic mean in arbitrary units, $\omega$ the value of the arbitrary unit in absolute units, $N\omega$ the total area under the curve in absolute units, $\sigma$ the standard deviation in absolute units. Comparing (10) and (6) and taking $x = (s - \beta)\,\omega$, we obtain

$$D = \frac{\sigma^2}{2t} \cdot \left(\frac{l - b}{l \cdot G}\right)^2. \tag{11}$$

According to statistical theory $\sigma = \sqrt{\mu_2^0}$ where $\mu_2^0$ is the central moment of the second order in absolute units. $\mu_2^0$ is determined by the equation

$$\mu_2^0 = \left[\frac{\Sigma(s_i^2 S_i)}{\Sigma S_i} - \left(\frac{\Sigma s^i S_i}{\Sigma S_i}\right)^2\right] \cdot \omega^2.$$

In equation (12) the first fraction in the square brackets is the second order moment, and the second fraction the first order moment. Denoting

$$\frac{(s - \beta)\,\omega}{\sigma} = y; \quad \frac{\sigma S}{N\omega} = F(y) \tag{13}$$

according to (10) we obtain

$$F(y) = \frac{1}{\sqrt{2\pi}} e^{-\frac{l^2}{2}}. \tag{14}$$

The Gaussian curve is plotted from values of $y$, $F(y)$ taken from probability function tables, and then the values of $s$ and $S$, after transformations (13), are plotted on the same curve. In this way the curves *1* and *2* on Fig. 3 were constructed.

Identity of the experimental and theoretical distribution curves indicates monodispersity, while deviation from the Gauss curve can be interpreted as a manifestation of polydispersity of the substance. Another characteristic feature of a monodisperse substance is that the diffusion coefficient $D_1$, calculated by the statistical method, is equal to the diffusion coefficient $D_2$ determined by the other method. For polydisperse substance $D_1/D_2 \neq 1$ and the experimental curve does not coincide with the Gauss curve. The coincidence of the two curves indicates the ideal nature of the diffusion process i.e. the absence of anomalous diffusion (due to the effect of concentration, charge of particles, polydispersity and so on). If there is considerable molecular interaction, leading to the development of a gel-like structure in the solution, then a skew line (Fig. 3, curve *3*) is obtained instead of a symmetrical curve.

## TWO CELL ARRANGEMENT

As we mentioned above, in order to carry out parallel control experiments for the determination of $D$ we constructed a system incorporating two cells *12* and *13* and using the same optical system (Fig. 4). The monochromatic light beam, after passing through the scale *3*, can pass either through cell *12* or through cell *13*. In the first position of the shutter *17*, which is shown in Fig. 4 by a solid half-circle, the beam reflected from mirrors *5* and *4* passes through the first cell and by means of mirrors *10* and *11* is directed into the camera and forms an image of the scale on the photographic plate *16*. The path of the beam is indicated by arrows. In the second position of the shutter *17*, which is indicated by a dotted line, the first system of mirrors is shut out and the beam reflected from mirrors *6* and *7* passes through the second cell *13* and by means of mirrors *8* and *9* is directed into the same camera. Thus, by rotation of the shutter we can exclude first one (*7*, *8*, *9*) and then the other (*4*, *11*, *10*) system of mirrors and thus, employing the same scale *3*, we can photograph in turn either one or the other cell.

VOL. 5 1960

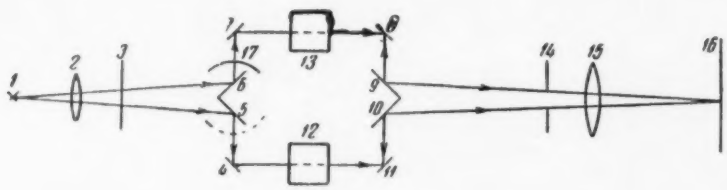


FIG. 4

The optical system and the mirror system are adjusted in such a way that the image of the scale, after passing in turn through the first and then the second cell, falls without distortion on the same region of the slit of the camera *16*.

All the optical units of the arrangement, except the mirrors, are similar to those which we used in the one-cell arrangement (Fig. 1). The mirror systems are attached on the front and back walls of the thermostat and are protected from dust by plastic boxes with rectangular windows of optically flat glass for entrance and exit of the beam. The photography of the scale image is effected automatically by an electronic circuit supplied from a voltage stabilizer.

## AUTOMATIC SYSTEM

The system (Fig. 5) operates in the following way: every 6 or 12 hr a clock, through a relay and magnetic starter, switches on the positive and negative stabilized voltage supply and the filament current. The negative voltage is applied to the grids of all the valves with the exception of the first. The positive voltage is fed directly to the anode of the first valve and to the anodes of all the remaining valves through a relay incorporated in the anode circuit of the first valve. The operation of the first relay breaks the circuit imposing the negative voltage on the grid of the second valve.

The condenser $C_1$, maintaining this voltage, begins to discharge through the resistance $R_2$ and after a fixed time, determined by the values $R_2C_1$. The condenser is discharged and the valve operates. This triggers the second relay, which switches on the magnetic starter, thus supplying voltage to the Ruhmkorff coil, the high voltage of which is used for supplying the starting pulse to the mercury lamp (SVDSh-250).

The operation of the second relay breaks the circuit imposing the negative voltage on the grid of the third valve. After a time, determined by the values $R_3C_2$, the third valve operates, triggering the third relay. It cuts out the magnetic starter of the Ruhmkorff coil and breaks the negative voltage circuit on the fourth valve. The remaining stages operate in a similar manner. The fourth relay provides a delay (10 min) between the onset of heating of the lamp and the start of photography. The

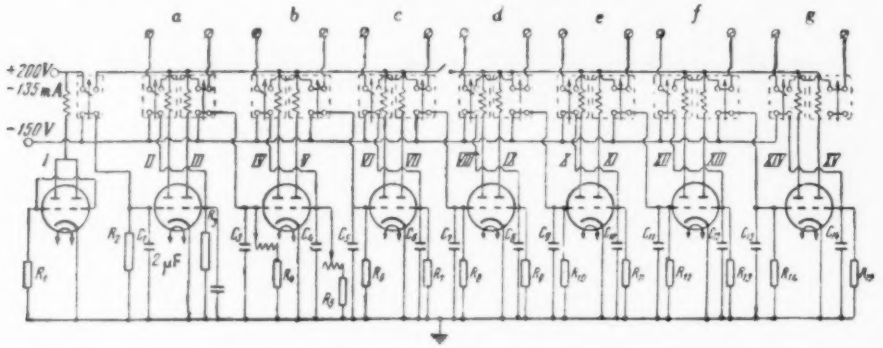


FIG. 5. Automatic system. (*a*) Ruhmkorff coil, (*b*) diaphragm shutter, (*c*) and (*f*)SD motor for driving plate, (*d*) and (*g*) SD motor for rotating shutter, (*e*) diaphragm shutter, SD for driving plate.

delay is required for the establishment of steady operation of the SVDSh-250. When the relay operates it opens the shutter, and photography begins. The fifth relay closes the shutter, i.e. it controls the exposure time (the exposure time can be varied from five seconds to two minutes by means of a variable resistance included in the grid circuit of the fifth valve. For the photographic plates which we used the exposure was 20 sec). The sixth relay switches on the motor. During a period of 27 sec the motor drives the plate 8 mm.

If the one-cell arrangement is being used, the system is automatically switched off after this. If the parallel two-cell arrangement is being used the seventh time relay is succeeded by an eighth relay, which operates the Warren motor activating the rotating shutter, thus stopping the beam going to cell *12* and opening the path to cell *13* (Fig. 4). The motor cuts out automatically as soon as the rotating shutter reaches the required position. The ninth relay disconnects the Warren motor driving the shutter. The tenth relay opens the shutter for photographing the scale when the beam passes through the second cell (*13*, Fig. 4). The 11th relay closes the shutter. The 12th cuts in the motor for moving the rotating shutter into its initial position, thus stopping the beam going to cell *13*. The 13th cuts out the motor. The 14th switches on the Warren motor

which moves the photographic plate for the next photograph. The 15th relay switches it off. On the completion of these operations the system is automatically switched off by the clock.

## CALCULATION OF MOLECULAR WEIGHT AND DISSYMMETRY COEFFICIENT

If we know the value of the diffusion coefficient $D$, the viscosity $\eta$, the partial specific volume $\nu$ and the dissymmetry coefficient $f/f_0$ of the particle, the molecular weight $M$ of the protein can be calculated from the formula

$$M = \frac{(RT)^3}{162\pi^2\eta^3 N^2 D^3 \left(\frac{f}{f_0}\right)^3 \cdot \nu}, \tag{15}$$

where $R = 8{\cdot}31 \times 10^7$ is the gas constant, $T$ is the absolute temperature, $N$ is Avogadro's number.

The value of $\eta$ is determined with an Ostwald viscosimeter, and the density and partial specific volume with a pycnometer.

To determine $f/f_0$ we must find the ratio of the axes $a/b$. The value of $a/b$ is calculated from the viscosimetric data from Simha's formula [9]

$$\left(\frac{\eta_{sp}}{\nu c}\right) = \underset{c\to 0}{\nu} = \frac{\left(\frac{a}{b}\right)^2}{15\left(\ln\frac{2a}{b} - 1{\cdot}5\right)} + \frac{\left(\frac{a}{b}\right)^2}{5\left(\ln\frac{2a}{b} - 0{\cdot}5\right)} + 0{\cdot}933. \tag{16}$$

W. Kuhn and H. Kuhn [10] suggested the equation for calculating the ratios

$$\eta_{sp} = 0{\cdot}6 + \frac{a^2}{5b^2}\left[\frac{1}{3\left(\ln\frac{2a}{b} - 1{\cdot}5\right)} + \frac{1}{\ln\frac{2a}{b} - 0{\cdot}5}\right]. \tag{17}$$

of the axes of particles having the shape of very elongate ellipsoids of revolution. Equation (17) is very similar to Simha's equation.

Determining $a/b$ in this way, we can calculate $f/f_0$ from the formula of Perrin and Herzog [11]. For elongate ellipsoids

$$\frac{f}{f_0} = \frac{\left(1 - \frac{b^2}{a^2}\right)^{1/2}}{\left(\frac{b}{a}\right)^{2/3} \ln\frac{1 + \left(1 - \frac{b^2}{a^2}\right)^{1/2}}{b/a}}, \tag{18}$$

Thus, by determining $\nu$, $\eta$ and $f/f_0$ experimentally and putting their values in (15), we can determine the molecular weight of the protein.

Knowing the diffusion coefficient and determining the sedimentation coefficient of the protein particle, we can calculate the molecular weight from Svedberg's formula [12]:

$$M = \frac{RTs}{D(1 - \nu \cdot \rho)}, \tag{19}$$

where $s$ is the sedimentation coefficient, $\rho$ is the density.

The ratio $f/f_0$ is calculated from the formula

$$\frac{f}{f_0} = 10^{-8}\left(\frac{1 - \nu \cdot \rho}{D^2 \cdot s \cdot \nu}\right)^{1/3}$$

*Translated by* F. L. SINCLAIR

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VOL
5
196

# AN AUTOMATIC FRACTION COLLECTOR*

V. A. STRUCHKOV

Institute of Biophysics, U.S.S.R. Academy of Sciences

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AN automatic fraction collector (Fig. 1) is described; many such instruments have been described* or made by foreign firms, but, all the same, this instrument is of interest, because it is simple and may be made up in most laboratories without great labour or expense from materials and parts readily available. It is adapted to analytical

VOL.
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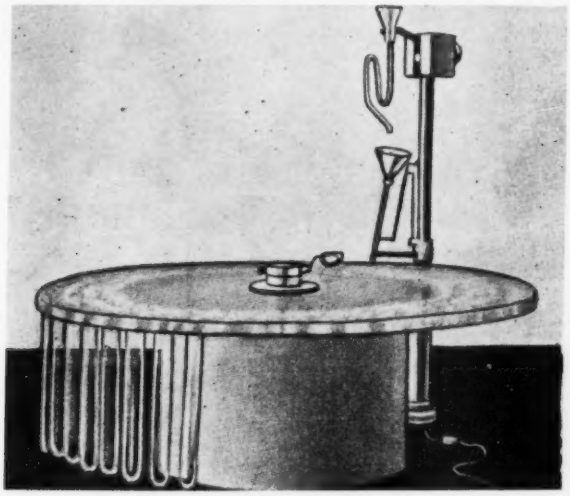

FIG. 1. General view of the collector.

and preparative uses. It may be controlled by time or by volume, and it takes 200 standard test-tubes in four rows on a rotating carrier. In preparative work the fractions may be collected in beakers on the bench (Fig. 2); in this case the rotating head carries a funnel. A mechanism of this type is found in a Swedish collector (the 3422).

The maltese-cross drive has two functions, namely to turn and to hold the head; a simple circuit with mercury switches can be used. A cycle consists of transfer to the next tube (working phase) followed by preparation of the switches for the next step (idling phase). During the working phase the motor *M* (Fig. 3), which is fitted with a reduction gear 6, drives the maltese-cross system, which consists of the driving

* Biofizika **5**: No. 1, 75–78, 1959.

† A very detailed list of such collectors, with brief indications of their parameters, is given in Chem. Listy **49**, 1087 (1955).

member *2* and driven member *1*, which are coupled to the carrier *C*. The pin drive *P* operates on the cross *K* and turns it through 90°C. (the pin drive turns through 360°). Therefore the working phase consists only of that part of the motion during which *K* performs a quarter-turn. The rest of the motion makes up the idling phase.

FIG. 2. Collector used in preparative work. *A* is the distribution funnel.

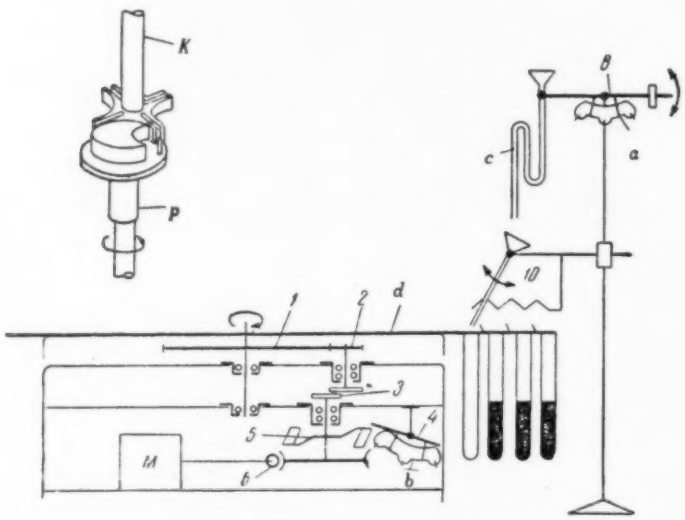


FIG. 3. The mechanical system. *a* and *b* are three-point mercury switches, *C* is the carrier (perspex, 8×500 mm), *1* the driven member (duralumin, 175 teeth, modulus 1), *2* the driving member (brass, 14 teeth), *3* the maltese-cross mechanism, 4 the rocking arm (carrying switch *b*), 5 the cam system, 6 a reduction gear (ratio 50 or 100), 8 the siphon arm (with switch *a*), and 10 the row-stepping device. *M* is a DAG-1 motor (1450 rpm) or a DBA-U4 one (695 rpm), *K* the maltese cross and *P* the drive pin (both in *3*).

In volume-controlled working the glass siphon *B* (Fig. 3) is balanced on the arm *8* by an appropriate counterweight. Two or three mercury switches perform all the operations. The first (*a*) controls the system; it is fixed to arm *8*, and sets the circuit

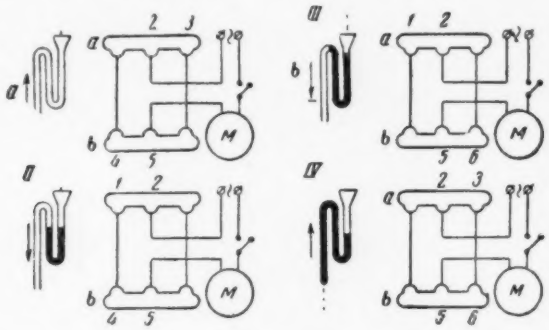


FIG. 4. Electrical system used in volume-controlled operation. I: Position of siphon at start; *2*, *3* and *4*, *5* are circuits, working alternately (*1*, *2* are controls of the working phase). II: Siphon half-full (*1*, *2* and *4*, *5* are the contacts in use when switch *b* is in position *5*, *6*). III: Siphon full; *1*, *2* and *5*, *6* are controlling the idling phase (switch *a* in position *2*, *3*). IV: The siphon discharges and rises; *2*, *3* and *5*, *6* control the second part of the idling phase (switch *b* breaks *5*, *6*, and so on). At *a* the siphon is at the extreme top position; at *b* it is at the extreme bottom position. The arrows indicate the directions of motion.

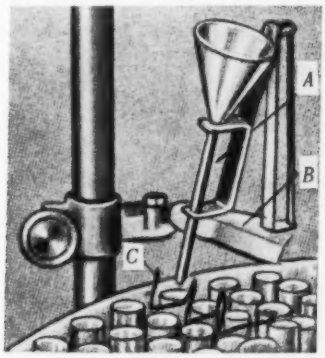


FIG. 5. Row-stepping device *A* is a spring funnel-holder (steel strip 0·5—10 mm); *B* is a plasm support fixed to the rod carrying the column; *C* is the deflector.

to working or idling, in accordance with the position of the arm. The second (*b*) switches off the drive and prepares the circuit for the step to the next tube; it is coupled to the rocking shaft *4*, which is acted on by the cam mechanism *5*. Fig. 4 illustrates the sequence of operations.

The tubes are filled as follows. The collector presents the next tube when the siphon becomes half-full, because the arm tilts, and the carrier turns (Fig. 4, I and II). As soon as the new tube is in position the cam mechanism *5* trips switch *6*. The instrument stays in this state until the siphon is full (Fig. 4, III). The fraction runs out, the arm

tilts, and switch *a* energizes the motor (Fig. 4, IV). The idling phase continues until switch *b* is again tripped by *5*; the circuit is then restored to the state existing immediately before the working phase (Fig. 4, I). The next working phase starts as soon as the siphon is half-full, and so on.

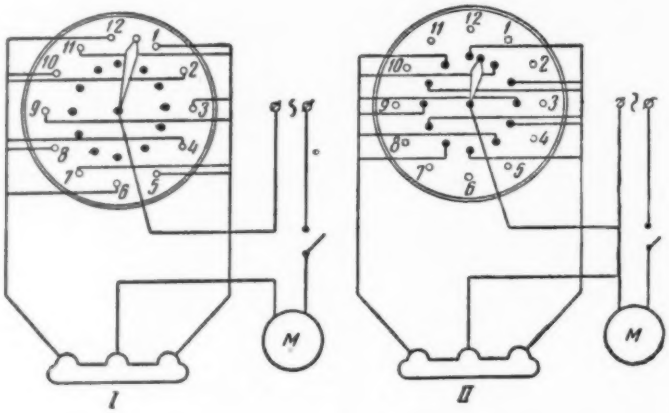


FIG. 6. Circuits used in time-controlled operation I: Contacts (with minute hand) used to collect fractions every 10, 15, 20, 30 or 60 min. II: Contacts (with hour hand) used to collect fractions every 2 or 4 hr.

The row-stepping mechanism *10* (Fig. 5) ensures that the rows are filled in succession. A device of this type is found in a Swedish collector (model 3413-A).

There are two modes of time-controlled operation.

(a) Here the operation is essentially analogous to volume-controlled operation; the siphon receives a controlled stream of water, and so acts as a time source. In this case either the single column feeds all four rows in succession, or four columns feed one row each.

(b) Here the collector is controlled by an alarm clock*, which replaces switch *a*. The clock operates the collector at set time intervals (the hand makes contact with pins on the dial). The time intervals may be varied from 10 min to 6 hr (Fig. 6).

The mechanism† is contained in a cylindrical box. The carrier is 500 mm in diameter and stands 180 mm above the bench.

It would be quite possible to use other materials and components, and to alter the number of rows or number of tubes in a row. The system might be simplified if the ball bearings were replaced by plain ones (except in the case of the reduction gear) and a ready-made (small) reduction gear of ratio 1:50 or 1:100 were used. The maltese-cross mechanism+ is to be found in any cineprojector.

Ribonucleotides and DNA have been separated by means of this apparatus.

*Translated by* J. E. S. BRADLEY

* The dial is replaced by a plastic disk having contacts.

† A. I. Khromov helped in making the mechanism.

+ Used maltese-cross mechanisms (obtainable from projector repairers) are quite suitable.

# DOUBLE-BARRELLED MICROELECTRODES FOR INTRACELLULAR MEASUREMENT OF BIOPOTENTIALS*

A. I. SHAPOVALOV

I. P. Pavlov Ist Medical Institute, Leningrad

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THE progress made in electrophysiology during the last decade is largely due to the discovery, and to the adoption in physiological studies, of the method of intracellular recording of the electrical phenomena arising in nerve and muscle tissue during their vital activity. In our country this method was first employed in Kostiuk's laboratory [1] and is now beginning to find application in other laboratories. The usual intracellular microelectrode consists of a glass micropipette filled with an aqueous solution of electrolyte. On being inserted into the cell the electrode is capable of measuring the resting potential of the cell, or the rapid fluctuations of potential pro duced on excitation.

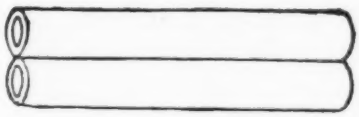

FIG. 1. Glass blank used for the pulling of double-barrelled microelectrodes.

A serious drawback, greatly restricting the applicability of this method, is the difficulty of registering the intracellular potential when the cell under observation is being acted on directly. It is even more difficult in the case of action at the site of insertion of the microelectrode. However, if the microelectrode is divided into two or more ducts it becomes possible to subject the investigated cell to a series of controlled actions at the site of insertion — to change its polarity, to introduce ions or pharmacologically active substances inside the cell, or to stimulate it electrically. This method was recently employed successfully by Coombs *et al.* [2] in studies of the motoneurones of the spinal column. The double-barrelled electrode used in their work was obtained by inserting a flat glass partition along the axis of the glass micro-electrode tube. This method of construction involves considerable technical difficulties and was not described in detail by the authors.

We have been using a double-barrelled intracellular microelectrode for measuring electric potentials from muscle fibres of the frog sartorius. These electrodes were

* Biofizika **5**: No. 1, 79–80, 1960.

prepared by pulling (with an electromagnet) glass blanks heated in a glowing platinum loop, as was described by Alexander and Nastuk [3] and Kostiuk [1]. However, instead of the usual single-bore tubes of these authors we used, as blanks, double tubes of refractory bero-silica glass, i.e. two tubes of outer diameter about 1 mm, fused together lengthwise (Fig. 1). These blanks were pulled in the flame of the usual benzene burner from tubes of larger diameter.

When the blank is heated in the glowing metal loop during the preparation of the microelectrode, a portion of the tube is melted and the electromagnet pulls and breaks both barrels simultaneously, and the tip of the blank acquires a sharp conical form. The length of the cone and its taper can be controlled, the degree of heating of the metal loop being varied by the pull of the electromagnet. A microscopic examination of the prepared electrodes with the aid of an eyepiece micrometer (AM-9-2) showed that the two barrels were separated over the whole length of the microelectrode, and

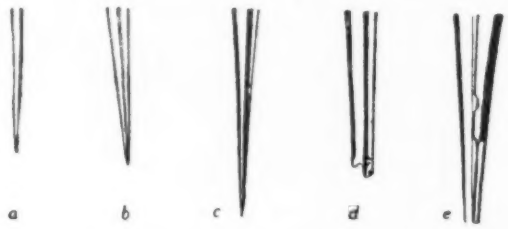


FIG. 2. Photomicrographs of capillary intracellular microelectrodes. (*a*) usual single barrelled electrode; (*b*) double-barrelled electrode with ducts of equal size; (*c*) double-barrelled electrode with ducts of different size; (*d*) double-barrelled electrode with broken tip; (*e*) double-barrelled electrode with air bubble in one of the ducts; magnification $10 \times 15 \times 0{\cdot}056 \times 2{\cdot}5$.

terminated in a common tip, the diameter of which did not differ from the outer diameter of the tip of the usual single-barrelled microelectrode. By regulating the degree of heating, we succeeded in obtaining double-barrelled micropipettes with an overall tip diameter 3 to 0·5 μ. Fig. 2 shows photomicrographs of electrodes.

The double-barrelled microelectrodes constructed in this manner were filled in the usual way by boiling in methyl alcohol, which was replaced in turn by distilled water and by 3 M potassium chloride solution. The resistance of each duct, measured by means of a shunted standard resistance [4], was 3–20 MΩ. When the diameters of both ducts of the blank were equal, the resistance of each barrel had a similar value, the difference between them being 1–3 MΩ. When the ducts were of unequal diameter, their resistances could be 5 and 15 MΩ, so that the resistance of one duct was 3–3·5 times greater than that of the other. However, the latter circumstance is not necessarily an obstacle to the application of the electrode, since each barrel can be used for a different purpose.

When the electrode had been prepared it was fastened rigidly in a special holder (Fig. 3) on the micromanipulator MM1, which provided motion in a vertical and

horizontal direction. One of the ducts was connected by a chlorinated silver wire to the grid of a cathode follower forming the input stage of a d.c. amplifier. This stage has been described in detail by Golov and Kostiuk [4]. Its low input capacitance (about 2·5 pF) permits the use of electrodes with very large ohmic resistance. The insertion of the electrode into the muscle fibre was performed under a binocular microscope MBS-2. The rigid attachment of the microelectrode, a feature of our method which differed from that of other authors, had the advantage that after insertion

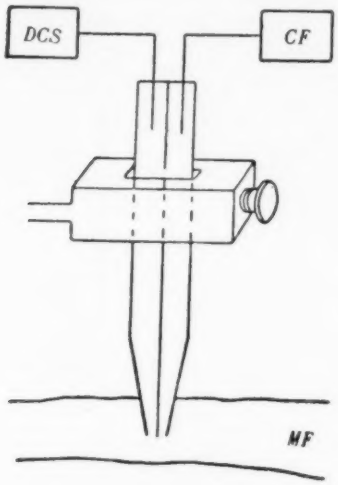


FIG. 3. Schematic representation for registering muscle fibre potentials with double-barrelled microelectrode. Symbols: *DCS*, d.c. source; *CF*, cathode follower; *MF*, muscle fibre.

of the electrode into the cell it was possible (without altering the position of the electrode and hence without subjecting it to the risk of breakage, or exposing the cell to traumatization) to connect the grid of the input stage to any of the barrels. This latter fact is of great importance since the checking of the intactness of each duct, which is carried out during the experiment by determining the resistance of the electrode, requires the connection of each duct in turn to the cathode follower.

In view of its small diameter the double-barrelled electrode penetrates just as easily into the cell as a single-barrelled one. The value of the resting potential, recorded in this muscle fibre by each of the barrels, was the same and was about 80 mV.

After several successive insertions of the electrode into the cell its tip was broken, this being indicated by the sudden drop in the resistance of the electrode. The break could be confirmed by examination of the electrode under the microscope.

In some cases the intactness of one duct might be destroyed without damage to the other. This occurred when only one of the barrels was broken, becoming several microns shorter than the other. The electrode could then be used as a single-barrelled one.

After insertion of the double-barrelled electrode into the cell and the checking of the resistance of its barrels, one duct, connected to the grid of the cathode follower, was then used for measuring the fibre potentials, and a silver, silver chloride electrode connected to a d.c. source was inserted into the other opening of the other barrel (Fig. 3). When necessary we could increase or reduce the polarization by means of this electrode, and at the same time we could register the resting potential of the cell. By filling the electrode with particular ions we could introduce them electrophoretically into the investigated cell. Using the method described we could also directly stimulate the muscle cell with electrical pulses by connecting one of the ducts to the output of a pulse generator. In this last case, however, special compensation for the stimulating current loop was necessary.

In view of the small diameter of the tip the double-barrelled microelectrodes described here can also be used for studying the activity of nerve cells.

*Translated by* F. L. SINCLAIR

VOL
5
196

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* There is a translation into English of this work.

# PRACTICAL NOTES

## INCREASE IN THE YIELD OF RADISHES AND CARROTS BY X-OR $\gamma$-IRRADIATION OF THE SEEDS BEFORE SOWING*

L. P. BRESLAVETS, N. M. BEREZINA, G. I. SHCHIBRIA, M. L. ROMANCHIKOVA, V. A. IAZYKOVA and Z. F. MILESHKO

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IN treating dried seeds of radish and carrot with ionizing radiation, the dose required to stimulate growth and development of the plants was established.

Dry seeds of the radish varieties "Saks" and "Red with white tip" were irradiated. In laboratory experiments, seedlings given 1000 r showed improved growth. When the results obtained were checked in field trials over 3 years these root crops gave

* Biofizika **5**: No. 1, 81, 1960.

increased yields of 11–33 per cent. At the No. 2 "Zarech'e" Vegetable Combine in the Moscow Region a commercial sowing of irradiated radish seed was made on an area of 5·1 hectares. The yield on this area was 11 per cent higher than on a control plot sown with non-irradiated seeds, this is equivalent to about 1000 kg more per hectare. The root crops from the seeds irradiated before sowing began to ripen 5–6 days earlier than the controls.

Experiments on preliminary irradiation of carrots were carried out over 3 years. The seeds irradiated were of the varieties "Shantane" and "Nantes". Seeds given 2000 to 4000 r showed increased germinating capacity. When grown in Petri dishes the roots of the irradiated seedlings were 20–24 per cent longer than those of the non-irradiated controls. Field trials showed a 26 per cent increase in yield with a 2·7 mg per cent increase in carotin content, resulting in a 57 per cent addition in the total carotin. When the results were tested under commercial conditions at the Krasnodar Vitamin Combine, the additional amount of carotin obtained on a 5 hectare field in 1957 was 35 per cent and on an area of 30 hectares in 1959 it was 38 per cent.

We also established the irradiation doses which increased the yields of other agricultural plants. These included rye, which requires 1000 r irradiation before sowing, peas, 500 r; cucumbers, 300 r; and tomatoes, 1000 r. In repeated trials all these plants gave increased yields under semi-commercial conditions.

The method of irradiating dry seeds of radish and carrot before sowing should be widely used in agricultural practice.

*Translated by* C. P. DUTT

VOL. 5 1960

# THE APPLICATION OF IONIZING RADIATION IN PLANT SELECTION *

V. V. KHVOSTOVA and S. A. VALEVA

Institute of Biophysics, U.S.S.R. Academy of Sciences, Moscow

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THE application of ionizing radiation in plant selection is based on the fact that irradiation causes irreversible changes, mutations, in plant cells. The altered cells are responsible for the formation of plant tissues and organs with a modified type of metabolism, which may sometimes be reflected in alteration of morphological characters. If cells that give rise to new generations of organisms are altered, the modifications will be inherited. The mutations caused by irradiation include a certain proportion of useful modifications of practical value for obtaining improved varieties.

* Biofizika **5**: No. 1, 81–84, 1960.

The method of irradiation selection principally depends on obtaining a great variety of forms to serve as the starting point for further selection. When plants are irradiated there is a marked increase in the number of hereditarily modified forms compared with the number of modifications arising under natural conditions.

By using ionizing radiation in selection studies, it is possible to solve a number of special problems. Thus, by means of irradiation it is possible in a comparatively short time to modify any particular character of a given variety, while leaving unchanged the complex genetic structure as a whole. It is possible, for example, to obtain a variety that is disease-resistant while retaining its other valuable qualities.

It is also possible through irradiation to break the connection between two characters that are inherited together, a separation which cannot be effected by any other known method. In oats, for example, it is possible to separate rust-resistance from liability to helminthosporiosis. By using irradiation in work on distant hybrids it is possible also to transfer particular characters from one parent form to the other, which cannot be done by other methods. By irradiating a hybrid of *Aegilops* and wheat, for example, it was possible to transfer to the wheat only the disease-resistant character of *Aegilops*.

The use of ionizing radiation is of special value in the selection of vegetatively reproduced plants. Irradiation of the buds produces a large number of modifications and the modified shoots can then be multiplied vegetatively.

The use of ionizing radiation in selection makes it possible to increase the diversity of the initial material for selection and to improve varieties by the production of new inherited modifications affecting particular characters.

It is noticeable that modifications of certain characters (strength of haulm, early-maturing, resistance to particular diseases) arise comparatively often in irradiated plants (3–7 cases out of 1000 ears of $M_1$).

*Irradiation.* For selection work by means of ionizing rays, sources of external irradiation (cobalt and X-ray installations, neutron-irradiation in a reactor) are most often used, although radioactive solutions (radioactive phosphorus, etc.) are also effective.

Plants (or separate plant organs) which have been treated with external sources of irradiation, or which have developed from irradiated seed, are perfectly harmless to persons working with them.

If the plants are reproduced by seeds, the most convenient method is to irradiate the dry seeds, since they can be transported and stored after irradiation, which can be carried out at a suitable time, and the seed afterwards used when required*.

Dry seeds of different plant species, and even individual varieties of a species, often differ considerably in radio-sensitivity. The irradiation dose is determined empirically. As a guide to the approximate determination of radio-sensitivity, one can

* It should be pointed out that prolonged storage under ordinary conditions of irradiated air-dried seeds reduces their viability to a certain extent; it is therefore preferable to store the seeds in closed containers with a reduced quantity of oxygen.

use the summarized information on the radio-sensitivity of plants (*Biophysics*, in course of publication).

Since increased radiation gives rise to an increased number of inherited modifications, it is usual to employ doses such that less than half of the plants survive and produce progeny at the end of the vegetation period. These are known as critical doses. They vary from 7 kr up to 100 kr for different crops. It is thought by some, however, that weaker irradiation is preferable since critical doses may give rise to several mutations at once in a single cell, which may mask one another and be difficult to detect. It is best, therefore, to use several different doses to irradiate the seeds of a single crop.

Germinated seeds can also be irradiated. Since the reaction of seedlings of various crops to irradiation by γ- and X-rays is more uniform than that of dry seeds and since much weaker doses (about 1–2 kr) suffice, seedlings can be irradiated in the locality, using medical X-ray installations for this purpose.

VOL. 5 1960

Irradiation can be applied not only to seed, but also to plant pollen, the inflorescence, buds and, in vegetatively reproduced plants, to tubers, rhizomes and cuttings. Vegetative organs and pollen are also more radio-sensitive than dry seeds. Doses of 1–7 kr are used for their irradiation depending on the plant species. Besides the methods of irradiation mentioned, in such cases it is convenient to use portable cobalt installations as well as special γ-fields.

*The first plant generation developed from irradiated seeds.* The symbols γ-1, *x*-1 or *n*-1 are used to denote the character of irradiation: γ-rays, X-rays, or neutrons. It is proposed to use $M_1$ as a general sign, where $M$ is any action giving rise to mutations. The germinating capacity of the irradiated seeds, determined in the coleoptile or cotyledon phase, is usually not different from that of non-irradiated seeds. With powerful doses of radiation, however, a considerable number of seedlings die.

The surviving plants are usually more or less inhibited, showing retarded growth (especially in the period before flowering); they often exhibit aberrant or monstrous morphological alterations, and they are often sterile or are much less fertile.

The morphological changes in plants which have developed directly from irradiated seeds ($M_1$) are usually not inherited; they are mostly what are known as "radiomorphoses". Hereditary modifications are very rarely observed in the $M_1$ generation since the majority of the mutations are recessive and only appear in homozygotes in subsequent generations. Hence, although it is worth while to select particularly valuable specimens in $M_1$ (e.g. those unattacked by disease, early maturing and erect forms, etc.) and to sow seeds from them separately in the following year, it should be borne in mind that many of the modifications will not appear in their progeny. As regards genetic structure, $M_1$ plants are for the most part "chimaeras", since particular shoots of a single plant develop from groups of cells in which modifications may have been caused by radiation and so the tissues of the $M_1$ plant are not identical throughout. It is necessary, therefore, to collect and sow in the following year the seeds from all the ears, pods, etc. of each plant, since the seeds from different shoots may be gen-

etically different. The seeds from all the plants must be collected, even from those which differ in no way from the controls.

To obtain second generation plants ($M_2$) of self-pollinators, seeds can be collected from each ear, panicle, pod, etc., and sown in separate rows. To save labour seeds are sometimes taken from half the ear or panicle (20 seeds from each one). In view, however, of the considerable labour and certain technical drawbacks involved in the method of sowing separate rows, it is also possible to sow the seeds obtained after threshing all the plants given a particular dose of irradiation.

Seeds collected from the most interesting $M_1$ plants should be sown in separate rows (by groups).

When irradiated seeds of cross-pollinators are sown, the $M_1$ plants must be isolated during flowering in order to preserve the modified forms. If, however, selection is for quantitative characters, it is possible simply to choose the most productive groups.

*The second and subsequent generations* ($M_2$, $M_3$ *etc.*). Selection of hereditarily-modified forms should, in the main, begin with the second generation ($M_2$). The bulk of inherited modifications usually appears in $M_2$, but they may also be exhibited in subsequent generations. To detect mutants of interest to selectionists, a large quantity of $M_2$ plants should be grown (preferably some tens of thousands, in any case not less than 10,000).

When sowing seeds separately from each ear or pod, a hereditarily modified recessive character resulting from irradiation should appear in a quarter of the plants in the row, but in practice there are usually fewer.

If a desirable modification is found among the plants in any row, seeds from all the plants of this row, whether modified or not, are sown the following year in order to check the inheritance of the character concerned. Whether a modification that has appeared is inherited can only be ascertained in $M_3$.

A variant of the above procedure for revealing and selecting mutations is to sow $M_2$ not in separate rows of seeds taken from one ear or pod, but as a whole crop of the mixed seeds from irradiated $M_1$ plants. In this case all the $M_2$ plants are closely examined and the deviating forms selected. Not all the seeds from modified $M_2$ plants should be sown. It is recommended that seeds be sown from only one or a few ears or pods, leaving the remainder in storage. Seeds from individual plants must be sown in separate rows to obtain $M_3$.

The following method can be used to detect hereditarily modified forms which show only slight morphological differences, but which can be of considerable agricultural interest owing to their altered physiological features. From each row of $M_2$ plants the most vigorous and prolific are selected and seeds from them are sown in one or two rows to obtain $M_3$, in which it is easier to detect the modified forms.

To reveal certain features, the environmental conditions must be such that deviations from the average will stand out particularly clearly. Ionizing radiation not only gives rise to sharply deviating forms, but also increases the range of variation of quantitative characters within the variety. It is important to notice these, perhaps small, deviations from the normal and to pick them out for further selection work.

VOL
5
196

It is particularly important to remember this last point when working with cross-pollinating species. When working with these, as many plants as possible with desirable deviations from the given variety or their seeds, should be selected in $M_2$. Hence there must be considerable initial material for selection.

If a hereditary modification that arises is of interest to the selectionist, then the new forms are multiplied in subsequent generations and their economic value tested as with the forms obtained by the ordinary selection methods.

*Work with irradiated pollen.* When irradiated pollen is used, each of the plants of the first generation grown from seeds which were set with irradiated pollen will consist of tissues with the same genetic constitution, i.e. it will not be a chimaera, as occurs with the irradiation of seeds. Therefore if a dominant hereditary modification arises, it can already be selected in $M_1$. Far more frequently, however, owing to the recessive character of the new mutations, it is only possible to detect the modified characters in the second generation after self-pollination, when it will appear in plants which are homozygotic for this character. The rest of the work is carried out as with irradiation of seeds.

VOL.
5
1960

*Work with vegetatively reproduced plants.* Work with irradiation of vegetatively-reproduced plants offers good prospects. The somatic cells altered by irradiation give rise to modified shoots, and later whole plants, and the new characters can be transmitted to subsequent generations by vegetative reproduction without undergoing the segregation observed in reproduction by seeds.

In working with vegetatively reproduced plants it should be remembered that when rhizomes, buds, bulbs and tubers are irradiated some of their cells are altered while others remain unchanged. When the shoot grows the normal cells usually divide more rapidly and produce tissues consisting of normal cells. When working with such irradiated material, therefore, one must be able to detect the altered cells and obtain shoots from them. Moreover these shoots may be chimaeras, i.e. contain tissues with differently modified cells which must also be separated. This is achieved by the method of mass clone formation. The greater the number of clones obtained the greater the possibility of singling out the modifications.

Successful clone formation can be achieved by forcing as many side shoots as possible, which can be done by repeatedly cutting off the uppermost buds. The greatest number of modifications can be obtained by forming clones from shoots arising from dormant buds, from cuttings that have already rooted and buds situated at the bases of side shoots. By repeatedly cutting off the side shoots it is possible to induce the growth of new shoots consisting of genetically homogenous tissue which is often of a modified kind.

For more successful work it is best to use large amounts of radiation, since with weak doses only a few of the tissue cells will be modified and it will be difficult to detect them. Experience shows that the irradiated tissue acts as a reservoir from which shoots carrying modifications can be singled out over a period of many years.

*Translated by* C. P. DUTT

## LETTERS TO THE EDITOR

# THE EFFECT OF IRRADIATION ON THE PERMEABILITY OF RADICLES OF *VICIA FABA* *

T. G. MAMEDOV

Biological Soil Faculty of Moscow State University

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VOL
5
196

THE secretion and absorption of mineral and organic substances by plant cells and tissues has been used by many authors to study permeability and the harmful effect of external factors [1–3]. Many authors [4, 5] consider that ionizing radiation increases cell permeability, others [4, 6] hold the opposite view. Trudova [7] found that X-irradiation in small dose had no effect on exosmosis from root tips of oats, whereas lethal doses increased it. Kuzin and Strazhevskaia [8] studied the dynamics of absorption and secretion of radioactive phosphorus by X-irradiated wheat seedlings; they established that the greatest change in permeability occurred in the first hour after irradiation. Vasil'ev *et al.* [9] studied exosmosis from the largest radicles of irradiated wheat seedlings; doses of 300 r, 1, 3, 5, 20 and 30 kr had practically no effect on exosmosis.

The comparatively small change in exosmosis even with large doses of irradiation of the order of $10^4$ r was regarded as proof of the radio-resistance of permeability penomena [8, 9]. Determination of the total quantity of mineral and organic matter secreted by the roots does not, however, give a complete picture of exosmosis since alteration of the composition and properties of the secreted substances is not taken into account. On the other hand, it is well-known that in the course of its normal vital activity the root system can exert a direct effect on its environment by secreting physiologically active substances [3, 10].

The present work was undertaken to study permeability changes in the cells of irradiated bean radicles. At the same time the effect of irradiation on the rate of growth of the plants was studied.

### METHODS

The experiments were carried out with the black Russian variety of *Vicia faba*. Bean seeds of uniform size taken from a single harvest were soaked in tap water for 24 hr and then transferred to damp sawdust, for germination, for 2 days. Three days after soaking, radicles of equal length were selected for irradiation (the length of the radicle at this time was about 2 cm).

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Radioactive cobalt was used as the radiation source (apparatus GUT-S-400). The radicles were irradiated in a moist chamber with a dose rate of 30 r/min. To study the effect of irradiation on the growth of the radicles, the seedlings were grown on filter paper in glass beakers and the length of the radicle measured daily. Permeability was determined by exosmosis, measured by the total amount of organic matter secreted and the redox potential and pH of the external water. For this purpose the seedlings were transferred to Koch dishes (of 50 ml capacity) filled with distilled water. The dishes were covered with polymethyl methacrylate disks with holes for the radicles. Each dish held 15 plants. All variants of the experiment were continued for 8–9 days. The water in the dishes was changed every 24 hr, the water in which the radicles were immersed being used for analysis. Besides distilled water, tap water and Knop's solution were used in the experiments. The results were very similar, hence the data given refer to experiments in which distilled water was used as the medium. The organic matter secreted was determined by the permanganate method [3]. The results were expressed in mgm of oxygen needed for oxidation.

VOL. 5 1960

The pH and redox potentials were determined by a P-6 potentiometer, using platinum and calomel electrodes. Redox potentials were determined in specially prepared vessels through which nitrogen, previously purified from oxygen, was passed for 5 min. The pH was determined by means of the quinhydrone electrode. Throughout the experiment the radicles were kept in a thermostat at 20°C. Each experiment was repeated 5–6 times.

## RESULTS AND DISCUSSION

Data in the literature and experiments carried out by us indicate that suppression of the growth of radicles irradiated with less than 240 r is reversible. The rate and time of resumed growth diminishes with increased amounts of irradiation.

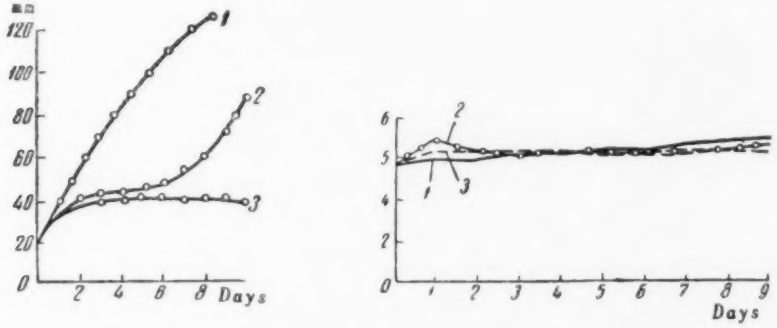


FIG. 1. Effect of irradiation on growth of radicles. (1) control; (2) 150 r; (3) 500 r.
FIG. 2. Effect of irradiation on the secretion of organic matter by the radicles. (1) control; (2) 150 r.; (3) 500 r.

Fig. 1 depicts the effect of partially lethal (150 r) and fully lethal (500 r) irradiation on the growth of the radicles. A perceptible lag in the growth of the irradiated radicles

begins on the second day after irradiation. The radicles given 150 r resume growth 6–7 days after irradiation but do not catch up with the controls. In the radicles given 500 r, not only is growth completely suppressed but considerable changes are observed; they become thickened and covered with brown hairs and the meristematic zone disappears.

In the second series of experiments, exosmosis of organic matter from the radicles was studied after the above-mentioned irradiation doses. The data obtained are shown in Fig. 2. The amount of root secretion in the control and experimental plants did not differ throughout the experiment, apart from an insignificant increase 1 day after irradiation. This confirms that exosmosis of substances from the radicle is not altered by ionizing radiation, as already noted in the literature [3].

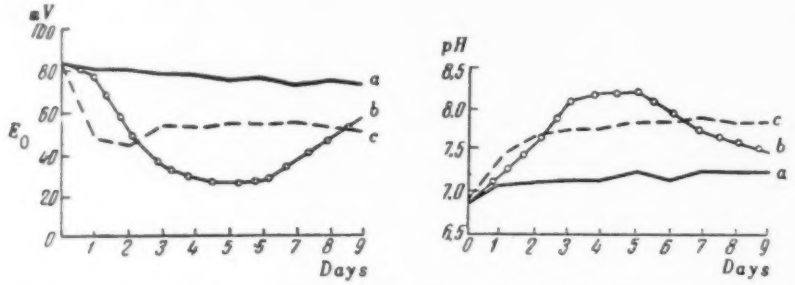


FIG. 3. Change of redox potential of water due to irradiated radicles. (a) control; (b) 150 r; (c) 500 r.
FIG. 4. Change of pH of water due to irradiated radicles. (a) control; (b) 150 r; (c) 500 r.

Figs. 3 and 4 give the results of experiments made to study the redox potential and pH of the water in which irradiated and non-irradiated radicles were grown. The data of Fig. 3 show that in the case of the non-irradiated radicles the redox potential is little changed throughout the experiment and remains at a high level, whereas for the irradiated radicles it is considerably lower. After 500 r the redox potential falls during the first day and thereafter remains at a low level until the end of the experiment. With 150 r irradiation the redox potential during the first day hardly differs from the control but it shows a marked fall during the following days. On the 5th day after irradiation, for instance, it had fallen from 82 mV to 22 mV. During the next 4 days, however, the curve rose close to the control.

Similar data were obtained in measuring the pH of the external water. As shown in Fig. 4, the irradiated radicles cause the pH to increase. With 500 r, the pH changed during the first day and thereafter remained constant. With 150 r the pH of the external water showed a still greater increase over the first 5 days but in the following 3–4 days it returned close to normal.

These data indicate that irradiated radicles considerably alter the pH and redox potential of the external liquid compared with controls.

If the secretion of organic matter is compared with the suppression of growth of the radicles (Fig. 1 and 2), it is clear that the alteration of intra-cellular metabolism

after irradiation is not reflected in exosmosis. At the same time, together with the growth of the radicles the redox potential and pH of the external liquid is correspondingly changed. The two irradiation doses, which differ in their biological effect, also evoke changes of a different character in the redox potential and pH of the surrounding water. A partially lethal dose of irradiation reversibly suppresses the growth of the radicles and the changes of redox potential and pH are also reversible. With a lethal dose of irradiation, however, where growth of the radicles is irreversibly suppressed, the change of redox potential and pH is also irreversible.

On the basis of the available data, it is difficult to explain why the alteration of redox potential and pH on the 4–5th day after irradiation is greater after a partially lethal dose than after a fully lethal dose.

Thus the changed pH and redox potential of the liquid surrounding the irradiated radicles reflects intracellular changes although the latter do not appreciably affect the total exosmosis of organic matter. Apparently a change in the balance of ions in the cells takes place as a result of irradiation. It is possible that in the course of it the pH within the cells is increased and weakly alkaline salts are excreted.

VOL. 5 1960

## SUMMARY

(1) In studying the basis of exosmosis changes in the permeability of radicles irradiated by γ-rays no alteration in the amount of organic matter secreted by the affected radicles was observed.

(2) Radicles given 150 r and 500 r irradiation were found to secrete substances causing a change (of 50 to 60 mV) in the redox potential of the surrounding medium and an increase pH.

(3) The alteration of the redox potential and pH of the external water by radicles given 150 r was reversible, but after 500 r it was irreversible.

*Translated by* C. P. DUTT

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* There is a translation into English of this work.

# NATURE OF PRIMARY CHANGES IN CELL RADIATION DAMAGE *

V. N. KOROGODIN and N. V. LUCHNIK

Faculty of Fundamental Biology, Moscow State University and Biophysics Laboratory of the Urals Branch, U.S.S.R. Academy of Sciences

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VOL
5
196

ALL the manifold reactions which occur in cells in response to ionizing radiations can, as a rule, be divided into two groups; damage based on localized changes in cell structures (recessive and dominant lethal mutations, chromosome breaks, translocations, etc.) and damage based on changes in the entire protoplast or a substantial part of it (radiation death, inhibition of mitosis, chromosome adhesions, etc). Damage of the first type is well explained by the target theory [1, 2], that of the second can be described in terms of the cumulative dose [2, 3]. Although the reversibility of cell damage indiced by ultra-violet radiation is well known [4], for the action of ionizing radiations, until quite recently, this was assumed only for cumulative effects appearing not later than the first mitosis [2]. However, a considerable volume of recent work has indicated that the recovery phenomenon also applies to other types of radiobiological reactions of the cells [5–9, etc†].

Quite independently of each other the authors of the present paper were able to obtain data suggesting the possibility of post-radiation recovery from cytogenetic and late cellular sequelae of $\gamma$-irradiation in experiments on yeast organisms and cells of higher plants.

The main results may be summarized as follows: the diploid yeast *Saccharomyces vini*, strain Megri-139-B was irradiated in the mitotic resting phase in sterile water and then plated out after at different intervals on Petri dishes or for the determination of the ratios of the various forms of damage on slides coated with nutrient agar. It was found that with an increase in the time interval between irradiation and plating the survival rate of the irradiated cells regularly rose [10] through the decreased number of cells inactivated after the first and after several cycles of multiplication [11]. The recovery rate depended slightly on the concentration of the cellular suspension and the oxygen tension, but decreased on lowering the temperature [12]. This is not in-

* Biofizika **5**: No. 1, 88–90, 1960.

† The number of articles devoted to problems of post-radiation recovery of cells has increased appreciably in the last year or two. The factual information and theoretical arguments advanced in these articles will be considered in detail in an article now in preparation. The arguments outlined in the present short communication are based almost entirely on an analysis of our own experimental findings.

consistent with the known increase in the survival rate following incubation after irradiation at sub-optimal temperatures [7], in view of the different experimental conditions.

The dried seeds of the Kanital variety of pea were irradiated with a dose of 15000 r and after cultivation the percentage of cells with chromosome aberrations in the first mitosis, which proceeded asynchronously and lasted a day was determined. All the first cells to undergo division were abnormal, whereas only 23 per cent of the last cells undergoing the first mitosis showed aberrations. Both the number of simple breaks and of asymmetrical recombinations decreased. This effect cannot be explained by elimination of the damage nor by the differing radiosensitivity in various phases of cell division. A similar decrease in the number of damaged cells was also observed after irradiation of the seedlings in the cells irradiated in the interphase, although here the recovery effect was less marked. On soaking the dried irradiated seeds in water, the rate of semi-recovery was 14·3 hr and on soaking in cysteine solution 7·5 hr. It was not possible to detect any fundamental influence by cysteine on the rate of recovery for the irradiated seedlings [13, 14].

VOL. 5 1960

The results of our work are described in detail in articles specially devoted to the experiments with the yeasts [10–12] and the pea [13, 14]. Here, we should like to put forward some general ideas arising from a joint consideration of the two mutually complementary groups of experiments (complementary in that for the pea it is easy to analyse the character of the cytogenetic changes, although it is much more difficult to assess quantitatively the death of the cells and the number of cell generations for which purpose yeasts are very convenient specimens).

In the case of chromosome mutations, we are dealing with a classical example of the localized action of radiations [2]. It is probable that the inactivation of yeast cells after irradiation falls within this category of effects [15, 16]. Therefore, one cannot extend the cumulative dose theory which explains recovery from retardation of the first mitosis [2] to the recovery effects observed in both groups of experiments. In relation to chromosome breaks, it has already been argued that the number of primary breaks is much greater than the number actually observed [2, 17–20 etc.]. An explanation of this phenomenon in terms of the target theory has been given by Lea [2] and although by now there are many papers which more clearly define and develop the classical scheme, the new point of view is only in the developmental stage.

According to Lea, genuine chromosome breaks occur directly by the action of irradiation. The sites of the breaks may join up and the longer the time lapse from the moment of the break and the further apart the two ends, the less the likelihood of their joining up. The re-union may be regular and cannot then be detected under the microscope or irregular corresponding to the visible picture of asymmetrical recombination. The physical and chemical mechanisms of re-unification and recombination are identical and their relative probability determined solely by space-time limitations: the longer the irradiation of the cells in the interphase, the higher the probability that genuine breaks will disappear. The decrease in the number of damaged cells must be accompanied by an increase in the relative number of asymmetrical recombinations.

Similarly it is difficult to explain the results of our experiments; since in the experiments with the pea, as pointed out above, the decrease in the number of damaged cells in time was accompanied by a decrease in the asymmetrical recombinations*. In just the same way Lea's hypothesis cannot explain the decrease in the late death of the yeast cells, especially in those cases in which only some of the progeny of the irradiated individual undergo inactivation [11, 16].

It seems that in explaining our results on recovery, one can adopt the following working hypothesis (which we advance only for ionizing radiations with low linear density of ionization, as all our experiments were with $\gamma$-rays; the mechanisms of the action of radiations such as fast neutrons and $\alpha$-particles is apparently different)

It may be supposed that the direct effect of irradiation is localized, but merely potential (latent), damage [6, 7] which, with a probability of less than unity, in a definite time and in suitable conditions may appear in the form of genuine damage. This probability is apparently unequally distributed over the nuclear cycle and has a clearly marked maximum before the start of division of the nucleus or in the early prophase. The probability of the appearance of primary potential damage may be influenced by the general state of the cells depending on vatious factors, including the cumulative radiation damage of the entire protoplast. It is here convenient to speak of the existence of a "resolving factor" determining the probability of appearance of potential damage The nature of this factor cannot as yet be identified, but one may speak of its considerable radiosensitivity and the fact that damage to this factor does not come within the category of localized effects. Primary potential damage, not yet having assumed a marked form, is capable of recovery and as shown by experiments with cells incubated after irradiation in the state of mitotic rest [10, 12], the probability of recovery rises with an increase in temperature and is only slightly dependent on time. It seems to us that the working hypothesis proposed may be adopted not only to explain the effect of recovery, but also in the analysis of the changes in cell radiosensitivity in ontogenesis, and such phenomena as the reversible factor of time and ultra-fragmentation [21]. It also covers the action of certain anti-radiation agents, in particular, the effect of using suitable substances following irradiation [13, 14, 22] and also individual cases of radio-sensitivity which is species specific.

With the new discoveries in radiobiology in recent years, there has been frequent discussion of the validity of the classical biophysical notions in this field of knowledge. The mutual exclusiveness of the so-called theories of direct and indirect action is, in our view, removed by the scheme of Lüning and Svenson [19, 20]. These workers in explaining the mechanism of the oxygen effect have postulated that both on direct and indirect energy transfer a whole spectrum of chromosome damage of differing severity arises, but the proportion of the weak damage is higher on direct transfer. In the absence of oxygen the severity of all the damage is diminished and the proportion of effective damage lessened. Our hypothesis, which must be regarded as

* Fuller arguments against the use of the classical scheme to explain the development, at least, of chromatid aberrations are given in another paper (Luchnik, Dokl. Akad. Nauk, in press).

VOL
5
196

complementary to the scheme advanced by these two workers, may reconcile the two theories, each with its own basis, and previously considered as mutually exclusive: the theory of the direct action of the radiation on the chromosomes [2, 23], and the theory of its action via changes in cell metabolism [24, 25].

Generally speaking, the mechanism of primary radiobiological changes and consequently the recovery mechanism are of a mixed character. In particular, the chromosome breaks may be due in addition to the causes proposed by us, to both the obvious damage by direct action and to the reaction of the nuclear apparatus of the cell to physical and chemical changes in its cytoplasm. The specific importance of each of such mechanisms may be determined by the physical properties of the radiation, the specific features of the specimen and the conditions of the medium. However, a detailed discussion of all three possibilities would go beyond the scope of the present communication.

In conclusion the authors take this opportunity of thanking B. N. Tarusov and N. V. Timofeyev-Resovskii for their interest in the work.

VOL. 5 1960

## SUMMARY

The article describes a working hypothesis according to which local damage to cells by ionizing radiations with low linear density of ionization may be of a potential (latent) character and the possibility of its appearance dependent on the cumulative action of the dose, the physiological state of the cells and the conditions of the medium. The hypothesis is used to explain the previously described recovery of diploid yeasts and cells of pea rootlets after irradiation.

*Translated by* A. CROZY

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# DETERMINATION OF SOME MOLECULAR PARAMETERS OF RIBONUCLEIC ACID (RNA) IN SOLUTION *

D. M. SPITKOVSKII

Institute of Experimental Biology, U.S.S.R. Academy of Medical Sciences, Moscow

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THE ever increasing amount of experimental work devoted to the study of the functions and properties of RNA makes it necessary to devise simpler methods capable of being carried out in any laboratory, but at the same time accurate enough for the characterization of preparations. In the present paper a relation between the molecular weight ($M$) and the intrinsic viscosity $[\eta]$ is derived, and this provides the mathematical basis of a sufficiently rapid, simple and accurate viscometric method for the determination of the molecular parameters of RNA.

In deriving the relation between $M$ and $[\eta]$ [1] for RNA we have used experimental data, from which it can be shown that the formula of Florey and Fox is applicable, at any rate empirically, to the RNA molecule. Naturally, in dealing with RNA we have introduced its characteristic parameters into the relation

$$M = \frac{[\eta]^2 M_c^3}{\Phi^2 b^6} \tag{1}$$

(where $M_c$ is the molecular weight and $b$ the length of the segment of the molecule of RNA; $\Phi$ is a constant $= 2{\cdot}1 \times 10^{21}$). The segment length was determined from

$$b = \frac{\overline{R^2}}{L} \tag{2}$$

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(where $\overline{R^2}$ is the experimental value of the square of the mean distance between the ends of the molecule; $L$ is the length of the fully extended molecule).
Accordingly,

$$L = \frac{M}{324} \times 3 \cdot 2\text{Å} \tag{3}$$

since 324 is the mean molecular weight of one RNA nucleotide [2] and 3·2 Å is the minimum distance between nucleotides [3]. Moreover, the number of nucleotides per segment ($p$) is, of course, given by

$$p = \frac{b}{3 \cdot 2} \tag{4}$$

Table 1 gives the molecular characteristics of RNA taken from the literature, together with our calculated values of $p$ for RNA with molecular weights of from $1 \times 10^6$ to $2 \times 10^6$.

TABLE 1

Values for some molecular parameters of RNA

| Published values | | | Calculated values | Published values | | | Calculated values |
|---|---|---|---|---|---|---|---|
| Reference | $M$ ($\times 10^{-6}$) | $\sqrt{R^{-2}}$ (Å) | $P$ | Reference | $M$ ($\times 10^{-6}$) | $\sqrt{R^{-2}}$ Å | $p$ |
| [4] | 2·0 | 900 | 13 | [5] | 1·7 | 670 | 8·5 |
| [4] | 2·1 | 750 | 9 | [4] | 1·92 | 800 | 11 |
| [4] | 1·75 | 775 | 11 | [6] | 1·0 | 600 | 11 |
| [4] | 1·58 | 810 | 13 | [7] | 1·1 | 550 | 11 |
| [4] | 2·02 | 765 | 9 | | | | |

Values of $p$ of the same order [1] can be obtained from the activation energy of RNA [9]. As will be seen from Table 1, for RNA the value of $p$ does not exceed 13 (in contrast to the minimum value of $p = 150$ for DNA [1]), which indicates considerable flexibility in the RNA chains compared with the rigidity of the DNA molecule. Knowing the value of $p$, we can find the value of $[\eta] = f(M)$ from the relation [1] and Table (1). Thus for RNA with $M = 2 \times 10^6$,

$$[\eta] = 5 \cdot 5 \times 10^{-4} \times M^{0 \cdot 5} \tag{5}$$

and for RNA with $M = 1 \cdot 0 \times 10^6$,

$$[\eta] = 4 \cdot 2 \times 10^{-4} \times M^{0 \cdot 5} \tag{6}$$

An increase in the coefficient $K$ with an increase in $M$ in equations (5) and (6) of the type $[\eta] = KM^{0 \cdot 5}$ is quite natural ,since

$$K = K_1 \beta^3 \tag{7}$$

(where $K_1$ is a constant and $\beta^3$ is a coefficient characteristic of the volume expansion of the molecule [1]). By putting equations (5) and (6) in logarithmic form, it is not difficult to find the function $K = f([\eta])$:

$$K = 5{\cdot}95 \times 10^{-4} [\eta]^{0{\cdot}403} \tag{8}$$

and finally the relation required

$$[\eta] = 3{\cdot}9 \times 10^{-6} \times M^{0{\cdot}64} \tag{9}$$

As will be seen from Table 2, equation (9) reflects the relation between the viscosity and the molecular weight of RNA with sufficient accuracy.

TABLE 2. COMPARISON OF PUBLISHED VALUES OF $M$ WITH VALUES CALCULATED FROM EQUATION (9) FOR RELATIONSHIP BETWEEN $M$ AND $[\eta]$

| Published values | | | Calculated values |
|---|---|---|---|
| Reference | $[\eta]$ (100 cm³/g) | $M$ (light dispersion) ($\times 10^{-6}$) | $M$ from (9) ($\times 10^{-6}$) |
| [4] | 0·725 | 2·0 | 1·9 |
| [4] | 0·82 | 1·9 | 2·2 |
| [9] | 0·80 | 2·0 | 2·1 |
| [4] | 0·715 | 1·78 | 1·85 |
| [4] | 0·756 | 1·94 | 1·97 |
| [6] | 0·362 | 0·6–0·8 | 0·82 |
| [6] | 0·4 | 1 | 0·93 |
| [6] | 0·425 | 1 | 1·0 |
| [7] | 0·4 | 1·1 | 0·93 |

Here it should be mentioned that the theoretical relationships (5) and (6) which we have derived are fairly close to that obtained experimentally by Thomas and Doty [10] for separate polynucleotide chains produced by division of the DNA molecule into two spirals of equal size. According to these authors, in this case $[\eta] = 6{\cdot}0 \times 10^{-4} \times M^{0{\cdot}5}$. It is important to note that $M$ was here determined by the method of light dispersion. The equation holds for $M \leqslant 1 \times 10^6$. Besides, as will be seen from equation (5) even for RNA with $M = 2 \times 10^6$, the coefficient has a value of $5{\cdot}5 \times 10^{-4}$, that is, it is lower than that found by Thomas and Doty for single polynucleotide chains with $M \leqslant 1 \times 10^6$. This may be due to the enhanced influence of hydrogen bonds with an increase in $M$, which, as it were, draw in the polymer molecule and reduce its size. This possibility, which we have shown by calculation, was pointed out by Thomas and Doty. Similar effects are known from experimental results obtained for a number of other high-molecular weight compounds [11].

It will also be noted that the expression (9) can be written in the form

$$M \approx 2{\cdot}82 \times 10^6 [\eta]^{1{\cdot}2} \tag{10}$$

which agrees well with the semi-quantitative relationship given by Girer [2] on the basis of a study of the properties of RNA.

$$M = \text{constant} \times [\eta][^{1\cdot2} \tag{11}$$

It may be thought that values for the viscosity of RNA ($M = 2\times10^6$) exceeding unity that have been obtained by some authors [2, 12–15] refer to a molecule somewhat deformed and with its system of hydrogen bonds destroyed. In some cases, instead of $[\eta]$ authors give values of $\eta_{sp.}/C$, which are usually slightly higher than the former.

Statements of this kind are based on a calculation from which it follows that for a viscosity of RNA ($M = 2\times10^6$) exceeding unity, the value of the coefficient $\alpha$ in the equation $[\eta] = KM^{\alpha}$ also exceeds unity; this is in no way characteristic of such a flexible molecule as RNA. Even for DNA $\alpha \approx 1$. In fact, recently the majority of authors have tended to the view that for RNA ($M \simeq 2\times10^6$), the value of $[\eta]$ approaches 0·8, which agrees well with the dimensions of the RNA molecule determined by the method of light scattering.

The viscosity of RNA should be measured (according to considerations presented by us earlier [1]) in solution with $\mu = 0{\cdot}1$ to $0{\cdot}2$ at the lowest temperatures possible in order not to destroy the system of hydrogen bonds [4]. The initial concentration of RNA in solution, when the absence of intermolecular interactions is taken into account, should amount to several hundredths of one per cent.

Throughout the present paper $[\eta]$ is expressed in 100 $cm^3/g$.

## SUMMARY

(1) It has been shown that high-molecular RNA is a relatively flexible polymer chain, the segment magnitude being of the order of 10 to 13 nucleotides.

(2) An empirical relationship has been derived between the molecular weight of RNA and its intrinsic viscosity:

$$[\eta] = 3{\cdot}9\times10^{-6}\times M^{0{\cdot}84}$$

which is the mathematical basis of a viscometric method for the determination of the molecular weight of RNA.

(3) The numerical values obtained suggest that an increase in the molecular weight of RNA is accompanied by an increase in the influence of hydrogen bonds, and this leads to a decrease in the effective size of the RNA macromolecule in solution.

*Translated by* E. HAYES

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VOL
5
196

# DISCUSSION

## CONCERNING AN ARTICLE BY L. M. CHAILAKHIAN — MODERN CONCEPTS OF THE NATURE OF THE RESTING POTENTIAL*

A. S. TROSHIN

Institute of Cytology, U.S.S.R. Academy of Sciences, Leningrad

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THE journal *Biofizika*, No. 4, 1959 published an article by Chailakhian [1] devoted to a review of the modern concepts of ionic asymmetry and the resting potential. The author sought to present his material as a neutral observer and devoted a special section to "a critical review of the main hypotheses on the origin of the resting potential". However, the polemic tone of this section imparts a controversial character to the article as a whole and the conclusions drawn by the author cannot be regarded as sufficiently objective and a true reflexion of the present state of the problem under discussion.

Chailakhian considers the former arguments advanced by the advocates of the membrane and phase theories of bioelectrical phenomena as merely of historical interest. He goes to the trouble of considering afresh the pros and cons of the two theories and seeks to convince the reader on the basis of an analysis of the contemporary experimental findings that the membrane theory is superior to the phase theory and fits the factual material available. However, the case presented by Chailakhian can hardly be considered persuasive. Let us take a look at it.

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(1) Chailakhian considers that the bulk (80–90 per cent) of the water and mineral substances of the protoplasm is in the free state and that the cells behave as osmometers. In support of this the author refers to certain papers [2–4] which refer to, as he writes, "a high osmotic activity of the excitable cells" (p. 394, Russian text). In his view this is also suggested by experiments with heavy water [5] which have demonstrated that all the water of the muscles is rapidly exchanged (within 40 min) for the water of the blood plasma.

At present no one doubts that the water molecules enter the cell at a high speed and are extruded from it at the same rate. Among other things, this fact does not fit the conclusions of the membrane theory on the chemical composition and structure of the cell membrane [6]. However, it is not merely a matter of whether the entire water of the cell is exchanged slowly or quickly for the labelled water of the ambient medium. In judging the state in which the water is present in the cells, it is important to discover whether the rate at which the total water of the protoplasm is exchanged for the water of the ambient medium is uniform. The relevant experiments with heavy water [7–9] show that a certain amount of the cell water (about 50 per cent) is exchanged for the labelled water of the surrounding medium at a rate which considerably exceeds the rate of exchange of the other part. This suggests that the water of the protoplasm is present in a state differing from that in ordinary aqueous solutions, for example, Ringer solution.

Chailakhian points out that the living cell cannot be likened to an ideal osmometer despite the fact that the bulk of the water and cations in the cell are present in the free state. He ascribes the deviations from the law of osmosis to the fact that the protoplasm possibly has a definite structure with mechanical elasticity as one of its distinguishing features, and to the fact that substances may enter the cell by means of active transport. However, no special corrections can explain the fact that the dehydrating effect of osmotically active substances increases at a rate proportional to the entry of these substances into the cell. According to the elementary laws of osmosis, the dehydration effect should in this case diminish. Incidentally, these facts obtained in work carried out some ten to twenty years ago represent a serious challenge to the membrane theory even in its present form.

In line with the sorption theory, the water of the protoplasm has reduced solvent capacity as compared with the water of the ambient medium. It may be assumed that the water in the protoplasm is in an organized form, similar, for example, to the solvate water in coacervate systems. This accounts for its low solvent properties. This conclusion is based not only on investigations of the mechanisms of the regulation of the water balance of the cells, but mainly on studies of the distribution of non-electrolytes and also organic and mineral ions between cell and ambient medium. Many workers [10–11] have established that the concentration of the dissolved fraction of the substances entering the cell in conditions of diffusion equilibrium is much less than that of the same substance in the surrounding fluid in equilibrium (i.e. the coefficient of distribution $C < 1$), the coefficient varying for different substances from 0·1 to 0·5). These findings are difficult to explain from the standpoint of the membrane

theory. Finally, it may be claimed that the cell membrane has its special pump for any given substance but such an assumption is purely hypothetical. The sorption theory does not require such a premise. According to this theory, selective sorption by the protoplasm of the given substance is due to the peculiarities of the structure of the protoplasm itself, which in turn is maintained by the energy of metabolism.

(2) In discussing the state of the mineral substances in the protoplasm, one thinks chiefly of potassium, since the amount of potassium exceeds that of all the other minerals present in the cell. Therefore, the essential role in formation of the resting potential has been assigned to potassium. A sharp controversy around this point has been going on for almost fifty years and it cannot even now be considered settled. The conclusions on the state of the potassium in the protoplasm based on the membrane and sorption theories are diametrically opposed. According to the first theory, 80–95 per cent of the potassium in the protoplasm is in the free state, according to the second 80–95 per cent in the bound form. The protagonists of the sorption theory have arrived at this conclusion on the basis of the following findings.

(a) Studies of the outflow of potassium from the cell into solutions of salts not containing potassium or into pure sucrose solutions have shown that in a very short time, a matter of a few minutes, a small proportion of the cell potassium (5–10 per cent) passes into the solution, within about an hour 20–30 per cent of the potassium has escaped while the remaining potassium content is retained by the cells for a long time (more than 24 hr) being extruded rapidly after the death of the cells. These findings have been obtained by many workers on isolated muscles and nerves and also by perfusing muscles in the intact organism [10, 11].

(b) In the preparation of acetone or alcoholic extracts of rapidly-frozen muscles only about one-third to one-fifth of the cell potassium passes into solution. If the muscles are first stimulated about half the potassium can be extracted [10, 11]. On precipitating crude solutions of actomyosine with acetone all the proteinous potassium is found in the precipitate. After preliminary denaturation of the protein, potassium is not found in the protein precipitate [12]. These experiments, despite the fact that they involved the risk of gross damage to the living cells, suggest that most of the cell potassium is in a bound form, most probably with the proteins, and that on excitation and damage to the cells the greater part of the bound potassium passes into solution.

(c) The presence of bound potassium in living cells is also indicated by the fact that the muscular volume decreases on excitation and that the curve of the change in volume with time corresponds exactly to the change in the action potential. This phenomenon can only be explained by the fact that during excitation reformation of ions occurs with the formation around them of solvate sheaths (electrostriction). The considerable decrease in the volume makes it necessary to ascribe this effect to the passage of bound and non-ionized potassium into solution. This phenomenon would not be present were the whole of the muscular potassium in a free form [13, 14].

(d) The curve of the dependence of the concentration of potassium in the cell on its concentration in the ambient medium in conditions of diffusion equilibrium also indicates that about half the potassium is firmly bound in the protoplasm. The

VOL
5
196

size of this fraction of potassium is not dependent on the concentration of potassium in the ambient medium (probably it is bound and non-ionized potassium). The second potassium fraction shows variation of the adsorptive type with the potassium level in the medium (probably here this potassium is bound by forces of adsorption and is in the ionized state). And finally, the change in the third fraction is directly proportional to the change in the concentration of potassium in the medium with a coefficient of proportionality of $C = 0{\cdot}45$. This is ionized, free potassium dissolved in the water of the protoplasm. These findings discount the notion that the greater part of the potassium and water is present in a free form in the cells [10, 11].

(e) Isotope studies of the rate of exchange of the intracellular potassium for that of the ambient medium also point to the presence of a considerable amount of bound potassium in the protoplasm. Such experiments have been performed on microorganisms [15, 16], ova of marine invertebrates [17], muscle [18–20], red blood cells of various animals [21–23], nerve cells [24, 25] and on isolated nerve fibres [26]. In most cases it was found that a small amount of intracellular potassium (5–10 per cent) was exchanged at a very rapidly, about half the potassium at a lesser rate and the remainder even more slowly. Were the total potassium free, the rate of exchange would be uniform. True, Chailakhian refers to two papers in which it was revealed by this method that 90–95 per cent of the intracellular potassium is found in the free state [27, 28]. However, the first work gave the end result of exchange after a very long time interval, or the results obtained with a severalfold increase in the external concentration of potassium (for speeding up the processes of potassium exchange). The second work was carried out on preparations of the isolated rat diaphragm and there is no doubt that in this case the muscle fibres were damaged.

(3) Chailakhian refers to the work of Hodgkin and Keynes [29] who determined the coefficients of diffusion and mobility of $K^+$ and $Na^+$ in the axoplasm of the giant nerve fibre of the squid and demonstrated that the coefficients for these ions in the axoplasm were the same as in aqueous solutions of the corresponding concentration. Chailakhian ends the paragraph devoted to this question with the words "Suitable calculations have shown that about 85–90 per cent of these ions are completely free in the protoplasm". (p. 395, Russian text). The reader will gain the impression that this conclusion follows directly from the factual data presented in the work. However, this is not the case. I would recall the technique of the work of Hodgkin and Keynes [29] to which the author refers.

These investigators introduced the radioactive isotope of potassium locally into the axon by immersing part of the fibre in a solution containing this ion. The solution was then washed off and the rate of migration of the radioactive isotope along the length of the fibre followed. In 3 hr, according to the authors, 14–55 per cent of the potassium of the axoplasm was exchanged for the labelled potassium of the ambient medium (this is the "free potassium" fraction which according to the sorption theory undergoes rapid exchange). The mobility and diffusion of this part of the intracellular potassium were also studied in these experiments. This is the gist of the technique used by Hodgkin and Keynes.

The evaluation of the results of this work by Chailakhian is by no means that of the authors themselves who state: "This does not prove that all the potassium inside the nerve consists of free ions since an appreciable fraction of the potassium might be bound so firmly that it is unable to exchange with the radioactive potassium which enters the fibre..." (p. 526 [29]). At the end of their article, they devote a special discussion to the problem of whether the behaviour of 14–55 per cent of the radioactive fraction may indicate the state of the total potassium within the cell and utilizing the results of other experiments [27] they conclude this may be possible. The latter work referred to is concerned with the kinetics of exchange of ions between cell and medium. Chailakhian himself classifies this work among those which "have produced rather contradictory findings" and the "discrepancies which are due to the probability of large errors in this method" (p. 394, Russian text). I have dealt with these papers above but it still needs to be pointed out that the conclusions of Hodgkin and Keynes on the free state of potassium in the cell are by no means as indisputable and convincing as Chailakhian presents them.

(4) In Chailakhian's opinion, the high specific electrical conductivity of the protoplasm in excitable tissues, which is only 1·5–3 times less than that of the surrounding medium, also indicates the free state of the potassium in the cells and thus conflicts with the sorption theory. But these facts show very good agreement indeed with the sorption theory. I shall give one example. According to our calculations, in the isolated frog muscle fibre the sodium and chloride ions make up the highest number of all the free mineral ions and their concentration in the sarcoplasm is 30 per cent of the external concentration. There are, in addition, to these ions also other cations and anions present in the free form. Consequently, the specific resistance of the sarcoplasm should not differ markedly from that of the external solution even if we take into consideration that the mobility of the anions and cations in the protoplasm may be lower than in the external solution.

(5) Chailakhian considers that the so-called cable properties of nerve and muscle fibres demonstrate the existence of the membrane and cannot be explained by the phase theory. This opinion is erroneous.

In fact, the widespread equivalent electrical scheme for the conducting fibres contains elements which may be interpreted as transverse resistance and capacitance of the membrane. However, we must not confuse the equivalent scheme reproducing only the electrical properties of a given circuit with its physical structure. Various physical circuits may correspond to one equivalent scheme.

We would recall that Herman, the founder of the cable theory of nervous conduction, suggested as a model, for the passive electrical properties of the nerve, a homogenous conducting rod placed in a homogenous conducting medium, the surface of which (geometric interface) may be polarized. Lorente de No [30] in the book quoted by Chailakhian writes: "Since the polarization at the interface plays the role of a thin layer of low conductivity interposed between two better conducting longitudinal conductors, fact 1 (spread of the electrotonic potential — A.T.) is readily explained by the theory..." (Part 1, p. 443).

VOL
5
196

Although the relevant section of the phase theory has not yet been worked out, it would appear that the basic premises of this theory help to explain the cable-like properties of nerve and muscle fibres.

(6) Chailakhian writes that "from the point of view of the phase theory it is quite impossible to explain values of the resting potential of the order of 60–90 millivolts" (p. 396, Russian text). It should be remembered that according to the phase (sorption) theory, the resting potential is formed anew at the border between the damaged and intact portions of protoplasm. The damaged protoplasm loses its phase properties and the bound potassium in it passes into a state of simple aqueous solution. Because of this phase and diffusion potentials arise at the border between the damaged and intact protoplasm which when summated give the resting potential.

Chailakhian states that the phase potential in these conditions cannot exceed 5–7 mV, since according to Troshin's findings [10], the coefficient of distribution of $K^+$ is only 1·5 times those of $Na^+$ and $Cl^-$. We feel that these arguments against the phase theory are based on a misunderstanding. None of the supporters of the sorption theory has ever claimed that the phase potential is formed on damage because of a difference in the values of the coefficients of distribution of $K^+$ and $Cl^-$ since at the site of damage both potassium ions and also its partner anions, e.g. the anions of protein, phosphororganic compounds and orthophosphates, etc, are liberated and not chlorine ions. Therefore the phase potential may be considerably higher than 5–7 mV. For the same reason the diffusion potential difference in the segment of intact protoplasm adjacent to its damaged area may be very large and its value in the main determines the resting potential measured experimentally. This derives from the fact that, according to the sorption theory, the bound potassium accounts for 80–90 per cent of the total intracellular potassium in intact protoplasm, e.g. in muscle fibres. The total amount of potassium may be freed on damage to the protoplasm with the result that the concentration of free potassium in the damaged area will be about 100 times greater than in the undamaged protoplasm. If all the ions of the liberated potassium were electrostatically bound to large organic anions, the mobility of which approximate to zero, the diffusion potential difference in these conditions would be approximately 116 mV. It is still necessary to add the phase potential to this diffusion potential.

Ling [31] measured the resting potential of frog muscle fibre and found it to be about 97 mV. The difference between the calculated and measured resting potential is obviously due to the fact that not all the potassium ions freed by damage to the sarcoplasm are electrostatically bound to immobile anions. About one half the potassium ions in these conditions will have as their partner orthophosphate anions, the mobility of which is considerably less than that of potassium. In addition, the magnitude of the diffusion potential difference will be influenced by the presence of other ions in the protoplasm. All this must contribute to a fall in the diffusion potential difference which follows from Henderson's formula [10, 11].

Thus, the value of the resting potential predicted by the phase theory approximates to that observed experimentally. Furthermore, according to Ling's hypothesis of

fixed charges [31, 32] (which Chailakhian rightly considers as a variant and practical expression of the sorption theory), the resting potential is calculated directly as the potassium potential according to the Nernst equation: the author supposes that the anions with which the potassium ions are bound are immobile (fixed).

(7) In the concluding part of the article, Chailakhian writes: "An important quantitative check on the membrane theory has been the investigations on the behaviour of the membrane potential with change in the external concentration of potassium..." (p. 396, Russian text). These are, in fact, very important but the results of the many experiments undertaken in this direction do not lend support to the membrane theory [33, 34]. The change in the external concentration of potassium, as shown in the paper by Chailakhian (Fig. 2, p. 397, Russian text), from 0 to 20 mM, i.e. with a reduction in the $K_{in}^{+}/K_{ext}^{+}$ ratio to several times less than the norm and with an increase in this ratio to an infinitely large value, leads to a change in the membrane potential which, however, does not correspond even approximately with the Nernst equation. Only with an increase in the concentration of potassium in the medium to more than 20 mM does the membrane potential change in conformity with the Nernst equation and behave as the potential of the potassium electrode. However, in these conditions damage to the cells is already patent. On introducing potassium ions and many other cations and anions into the cell there is no agreement between the values observed for the resting potential and the expected value [33].

Stephenson's work [34] is of great interest in this respect for he was able to show that with a very significant change in the ratio of potassium and sodium concentrations in the frog muscle fibre and during restoration of the former ratio, the resting potential behaves in a manner not compatible with the membrane theory: when it ought to show a fall it remains unchanged and when the resting potential should rise it, in fact, drops. The assumption of Lorente de No [30] that the cause of the fall in the resting potential on increasing the external concentration of potassium must be the direct effect of the potassium on the protoplasm and not a reduction in the $K_{in}^{+}/K_{ext}^{+}$ ratio is correct. The experiments of Huxley and Stampfly [35] in which it was demonstrated that the change in membrane potential occurs within 1–2 sec does not contradict the assumption of Lorente de No since this interval is sufficient for the potassium to manifest its action on the test object.

All the foregoing remarks on the article by Chailakhian show just how far the problems dealt with by him are from being finally resolved.

It must be again stressed that many of the phenomena which have been described in terms of the membrane theory, have not yet been considered from the standpoint of the phase theory and this applies, in particular, to certain aspects of modern electrophysiology. On the other hand, the phase theory is based on abundant experimental findings on the problems of cell permeability. This information is in fundamental contradiction to the basic tenets of the membrane theory.

Our personal work and an analysis of the findings in the literature convincingly show that the phase theory is based on principles which are in good agreement with

VOL
5
196

the wealth of experimental findings and that it provides the foundations for a general theory of distribution of substances between the cell and ambient medium and explains the mechanism of bioelectrical phenomena.

*Translated by* A. CROZY

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VOL. 5 1960

# BASIC PROBLEMS IN THE STUDY OF CO-ORDINATION OF VOLUNTARY MOVEMENTS IN MAN FROM THE VIEWPOINT OF BIOPHYSICS *

L. V. CHKHAIDZE

Georgian Institute of Physical Culture, Tbilisi

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VOL
5
196

THE study of the laws governing the control of voluntary movements in man and animals is of very great theoretical and practical importance. The information obtained may be of fundamental significance for the elucidation of problems connected with the construction of self-regulating mechanisms or for the solution of questions of training and performance of complicated movements in particular conditions.

Up till now, if we leave out of consideration Bernshtein's work [1–4], which has not received general recognition, these investigations have been made mainly from the physiological aspect, and very little biophysics has been involved in the treatment of the problems.

This neglect of the physical aspect of the question is quite inadmissible. All voluntary movements of living beings proceed not only with fullest regard to, and in conformity with, all the internal and external physical factors, but it is precisely for this and because of this that there has been created in the process of evolution a bone–muscle apparatus and that complex and clearly differentiated system of control for it, which we call the brain.

There is now a marked increase of interest in the study of these questions from the physical aspect. This is due not only to the rapid development of methods of study, but also to the demands of modern science and practice — cybernetics, cosmic physiology, the physiology of work and sport, and so on. It is to be expected that the accumulating information, combined with physiological observations, will provide better answers to the problems than has been the case so far.

This article is an attempt to throw light on those basic questions of movement control which lend themselves to study from the biophysical viewpoint.

Any voluntary movement of the parts of the body of a living being is effected by a sequence of actions of a particular group of muscles. This sequence is elicited by the conditions of the problem and the general field of movement and is capable of endowing the particular parts with the necessary acceleration and the required trajectory. In view of the now generally accepted principle of nervous action, we can state definitely

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that this is achieved by a strict sequence in the work of the nerve cells (or their groups) controlling the particular muscles.

It is obvious that in this case there is established a simple correspondence between the state of the nerve cells and the excitation of the muscles (isomorphism). This process as a whole is called information transfer, the general principles of which are the subject of *cybernetics*. Hence in what follows we will use its terminology, as best reflecting the nature of control, although we do not, of course, identify the living organism with an artificial mechanism.

A fundamental feature of motor acts of living creatures, from the biophysical viewpoint, is the exceptionally large number of degrees of freedom in the parts of the limbs. In man, for instance, the number of degrees of freedom for the end of one finger relative to the thoracic cage amounts to 12 [3]. If we bear in mind that mechanical systems with only three degrees of freedom present a very complex problem of control, it becomes obvious that in the case of movements of a living organism the whole central control system, which must be so constructed that it can at the required moment suppress the superfluous degrees of freedom of the parts and release the necessary ones [3, 4], solves an extremely large and complex problem. The question is complicated even more by the fact that, as many investigations, including our own [5, 6], have shown, not a single, stabilized movement proceeds without the participation (direct and positive) of corresponding reactive forces. Their correct utilization also requires control by the central nervous system.

This, however, is not all. It has been found [7] that the relation between the muscle tension and the resulting movement is not only not a single-valued one, but even for a simple flexion in one joint it must be expressed by a differential equation of at least the second order, and in two or more joints — by correspondingly higher orders. If, in addition we consider that usually not only the individual muscles, but entire groups of them operate for the most part in an auxotonic state (i.e. in conditions of some preliminary tension), then we have a fairly clear idea of the very broad complex of problems which must be solved (and solved successfully) by the central nervous systems controlling the movements of man and animals.

Thanks to the teaching of Sechenov and Pavlov we now have a knowledge not only of several of the most important principles of operation of these systems (conditioned reflexes, stereotypy etc.), but also of the functional side of the question (excitation and inhibition [8, 9]). A great deal of attention has been given by physiologists to this aspect of movement control, and a considerable body of factual information has been amassed.

We will attempt, however, to introduce a number of more precise concepts to the general questions of movement control.

The most fundamental feature in the mechanisms controlling the movements of living beings is, in our opinion, the presence of a *control ring*, i.e. information transfer not only from the controlling section to the controlled (*direct communication*), but also from the controlled to the controlling (*feedback*). This follows from the basic inability of the limbs for self-regulation and the impossibility of performing voluntary

movement without constant control by the central system. This has been experimentally corroborated by numerous examples of the disruption of motor co-ordination when the visual or proprioceptor senses are put out of action [3, 4].

We may assume that a portion of the direct communication is: brain — efferent nerves — muscle synapses — muscles — limbs. A portion of the feedback can be divided provisionally into two parallel pathways: the *external feedback*, in which the transfer of information takes place partially outside the organism, through the external receptors, and the *internal feedback*, in which the information passes wholly within the organism, through the proprioceptors. We can accordingly distinguish an *external* and *internal control ring*. The first effects control over the voluntary part of the movement, and the second over its synergetic elements.

The higher divisions of the central nervous system, according to all our information, are associated more with the external control ring, while the lower divisions are associated with the internal.

This is clear, firstly, from the fact that the higher divisions of the central nervous system have no direct communication with the muscle periphery, whereas the lower divisions completely dominate it, and, secondly, from the whole process of mastery of an unfamiliar motoraction. When an unfamiliar movement is performed the control over the specific synergetic elements, owing to the unpreparedness of the lower divisions, is delivered over to the higher divisions, which can effect this task only by employing the external receptors (i.e. the elements of the external feedback). The higher divisions, thus fulfilling two functions, one of which is not natural to them, effect control in a primitive form [3]. The fully co-ordinated movement occurs only when control over its meaningful part (defined below) passes over to the external ring, and the control of details to the internal. This permits the execution of the movement in its most perfect and economic form [4].

The question of the separation of the control rings is correlated with the question of movement *programming*. By the latter we have in mind the establishment of a definite pattern, necessary for the correct execution of the movement, in the work of the nerve cells.

As a first approximation we can distinguish two stages of this programming. The first, consisting of the solution of problems associated with the direction, time and velocity of movement (i.e. with its *meaningful* part), apparently devolves on to the higher divisions of the central nervous system, and they can be provisionally called the *assigning mechanism*. The second stage, which determines the sequence of the particular muscle synergies and other details, is performed by the lower divisions, which are justifiably called the *programming mechanism*, since they fulfil the function of this concept as it is understood in cybernetics.

The existence of such a division is partially proved by the fact that all the higher animals, including man, when deprived of the higher divisions of the brain (for instance the cerebral hemispheres) through trauma or other cause, are completely incapable of solving the meaningful problems of movement, but easily perform even fairly complex automatic movements, if they have mastered them before trauma, i.e. if the programme

of movement is already laid down. On the other hand, we know [3] that pathological changes in the conducting pathways of the internal feedback (for example, degeneration of the posterior roots of the spinal cord) lead not only to disorder of automatisms, but also to the impossibility of correct execution of a movement without visual control, while its meaningful part remains unaffected. Whence it follows that the activity of the assigning mechanism is associated more with the external control ring, and the programming with the internal, since in the latter case the movement is performed when the whole internal control ring is excluded. The flexibility of these mechanisms determines the quality of work of the entire system, and the proper distribution of functions between them depends on it.

If we compare the level of present knowledge of the activity of the assigning and programming mechanisms, it is quite plain that some aspects have been studied more than others. While the general theory of conditioned reflexes can provide the initial information for drawing up an algorithmic* description of these mechanisms, we must realize that a very great amount of work still confronts us in this sphere. Moreover, very little study has been given to the connexions between these mechanisms, particularly their role in the control of movement as a whole and of its parts.

VOL. 5 1960

The study of this whole complex of questions is the great, primary task of physiology and biophysics (biomechanics).

When the corresponding divisions of the central nervous system have worked out (wholly, and in parts, and with great rapidity) the programme of movement, its execution begins. For this, the muscle periphery is brought into a state of excitation. The excitation is effected by definite nerve impulses, having the nature of a brief volley of electric potentials, flowing with great rapidity through the nerve axons. The potential waves are transmitters of information from the brain to the muscles by means of some *code*. At a later stage of movement control the information may be transmitted in another way, in the form of electrochemical reactions (from synapses to muscles), light or air vibrations (necessary for action on the external receptors) and so on. It is quite plain, however, that in all cases this information acts as a stimulus for those reflex arcs which, in fact, are the control rings. In view of this we can give a broader definition to a stimulus — "*controlling information*", understanding by this all the external and internal factors acting on the controlling and controlled system.

Hence, not only the programming of the movement, but all the processing of the information also proceeds by coded signals. So far the basic nature of the coding of these signals has received little been study. We can only say that the entire control of movement is apparently built up on the employment of the so-called "*binary system*" (the "all-or-none" law). It should be noted that this system is theoretically the most interference-free of all the possible systems for such purposes.

Thus, we arrive at the following, probably the most important, task of biophysics (bioelectrics) — to find ways of deciphering these codes. The problem is exceedingly

* Algorithm — a set of rules fulfilled in a certain order in the programming of the work of a controlled system.

difficult and is hardly likely to be solved without the assistance of considerable mathematical equipment.

The power of the nerve impulses emanating from the brain is measured in millionths of a watt. At the same time the power of the muscle units brought into movement is many times that of the impulses and is of a mechanical nature. It is obvious that for the solution of the problems of conversion and amplification of these impulses there must exist an apparatus which, by analogy with an artificial mechanism, can conveniently be called a "*servomechanism*". An important part of this mechanism is the spinal cord and, perhaps, some of the lower divisions of the brain. The effects accomplished here, such as the transformation of the excitation rhythm and the deciphering of the signals, deserve very thorough study. The task of biophysics and physiology is to establish the corresponding laws.

Part of the servomechanism consists of the muscles, which are excited at the neuromuscular junctions where the electrical energy of the impulses in converted into mechanical energy of the muscle fibres. This conversion is of a very complex nature, and some essential details are still obscure. The analysis of this conversion is also a task of biophysics.

When the excitation of the muscle periphery and the contraction of the muscles takes place, the effector task of control of the movement is completed and the solution of the second part — the afferent, control over its course — begins.

The gathering of primary information on the muscle tone is carried out by the muscle proprioceptors, and about the joint angles by the tendon proprioceptors [4]. This information passes to the *collating mechanism* of the internal feedback. This is the name of the section of the controlling system where the information from the feedback system meets the command information. This section elaborates new correction-signals, which then pass again into the control circuit of the direct communication and give precision to the course of the movement.

The best solution of the differential equations of the movement actually takes place here. In other words, the collating mechanism realizes the *useful effect* of feedback.

There is probably a separate collating mechanism for the external and internal feedback. But while its localization and functioning for the external feedback belong more to the field of psychology and are not considered by us, the activity of the collating mechanism of the internal feedback may be studied with the aid of certain pathological disorders. In particular, in certain afflictions of the cerebellum (for instance, disseminated sclerosis) intention tremor is seen, very reminiscent of the fluctuations of a mechanical system which occur in cases of mismatch of the stabilizing signal in the collating mechanism of such a system [10]. This sheds light on the localization of the collating mechanism of the internal feedback. As regards its activity, we can surmise that this mechanism also effects the reception of the information (required for the solution of the differential equations) on the velocity and acceleration of the parts of the limbs, since the tendon receptors are sensitive only to the position of the parts in space [4]. This reception is effected by a comparison of the signals sent at a particular moment of time with the signals which occurred shortly before

this moment. From certain indirect data we may suggest [4] that the size of the time interval between the comparisons is 0·07–0·12 sec and determines the resolving time in the work of the collating mechanism.

In addition, we should point out that in this region the control rings are divided into a number of small parallel circuits, but others carry more condensed information. This makes it possible to predict a *signal-synthesizing* apparatus. All this must be a subject of thorough study in the sphere of physiology and biomechanics.

In conclusion we consider it necessary to dwell, as far as is possible within the framework of the present article, on certain specific questions.

We can possibly distinguish a "*motor memory*" as a special information-storing mechanism, connected with other mechanisms directly effecting the control of movement. The existence of this apparatus does not seem to be in doubt. Yet its localization raises great doubts, since we know of no disorganization of the motor memory without damage to the functions of the assigning and programming mechanisms, which in all probability themselves possess the ability to retain the programme of movement in the form of relatively stable connexions. However, we distinguish this unit as a possibility for certain specially complex sets of movements.

As regards the transducers of the external feedback we should note that this question has been better studied, but present knowledge is notably onesided. We know not only the conducting pathways of these receptors, but their function also. It has been established that their signals have the same coded character as in the other transducers. Hence we can take it that our sense organs are coding devices, converting information from the external world into information capable of being dealt with by the nervous system — bioelectrical impulses. However, the activity of the coding and deciphering mechanisms is still completely obscure and the treatment of these questions must also constitute one of the tasks of biophysics (bio-optics, bioacoustics, etc.).

We must also mention the special mechanisms serving for the suppression of "noise" and all other interference peculiar to control systems. Despite the fact that the existence of such mechanisms would appear to be indisputable, in view of the exceptionally high stability of our nervous system and its protection from interference, nothing is known either of their localization or their modes of functioning. We need not, in any case, imagine them in the form of entire organs or apparatuses — they may have forms unfamiliar in the present state of our knowledge. It might be postulated that excess of information (duplication of functions by many cells) and the vicarious properties of the nerve nodes play a definite part in this problem.

We now sum up. As we see from a consideration of our proposed scheme (possibly largely hypothetical), there are a very large number of uncharted areas in the problem of the control of voluntary movements in man and animals. In fact, we still cannot even establish any definite regular features in the functioning of individual units of the scheme, as is possible for artificial systems.

Without underestimating in any way the value of the large amount of work performed by generations of physiologists, we may nevertheless assert that a time has

come when neighbouring sciences — physics and mathematics — must be called in for the solution of these very important questions. Only the combined studies of a large team of persons working in many fields, can find a way to the solution of the problem, the basic questions of which we have tried to clarify in the present article.

*Translated by* F. L. SINCLAIR

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* There is a translation into English of this work.

## BOOK REVIEWS *

**E. ERNST** (Dir. d. Biophysik Inst. Univ. Pécs). *Die Muskeltätigkeit.* Versuch einer Biophysik des quergestreiften Muskels. Verlag der Ungarischen Akademie der Wissenschaften. Budapest, 1958.

Any attempt to look forward and delineate routes to the synthesis of knowledge accumulated by different branches of science is of special value in modern biology. In this respect the work of Professor Ernst, Director of the Institute of Biophysics at Pécs University (Hungary), is of interest. The author has occupied himself with the problem of muscle contraction for more than 40 years. He discovered such important facts for the theory of muscle contraction as the reduction in muscle volume during excitation, the exchange of $K^+$ and $Na^+$ in the working muscle, the absence of stored elastic energy in a contracted muscle under tension, and reduction in muscle volume with stretching; he also put forward a hypothesis on the crystallization of muscle proteins in the contraction process. Unfortunately, some of these discoveries are sometimes unfairly ascribed to others.

* Biofizika **5**: No. 1, 103–105, 1960

Professor Ernst's book "Muscle Action" is based on the author's own work and that of his close collaborators. The first part is entitled "The Structure and State of the Elements from which Muscle is Built Up". Problems of the structure of muscle fibres and fibrils at rest and during contraction and the aggregate state of myosin and actin are considered in this first part. The second part "Muscle Mechanics" is devoted to problems of muscle excitation, special attention being paid to a semi-conductor hypothesis of excitation. The behaviour of myosin during stretching and under pressure as well as thermodynamic problems of muscle contraction are also dealt with, and current ideas on the nature of this process are considered.

We shall consider some questions of fundamental interest. In the section on the structural basis of muscle contraction the author comes to the conclusion that the smallest functional unit of skeletal muscle that responds to electrical stimulation is represented by a separate fibril sarcomere. This conclusion is based on an experiment in which one of the author's co-workers succeeded in effecting stimulation of a single fibril and made a micro-cinephotograph of this phenomenon. Until recently the electrical stimulation of a single fibril was considered to be impossible and thought not to be in accord with the main principles of the muscle-excitation process. Further successful studies in this field would obviously change our general view on the nature of the development of muscle excitation.

VOL. 5 1960

The section on the state of the water and potassium in muscle cells deserves great attention. Up to now these questions have given rise to great disagreement among research workers. The advocates of the membrane theory consider that nearly all the $K^+$ in the cell is free. This view is supported by the following experimental facts. (1) A potential difference, in accordance with the Nernst equation, exists between the cell contents and the external medium. (2) The mobility of $K^+$ in nerve cells, measured by the isotopic method, is close to unity. (3) The electrical conductivity of the axoplasm of the giant axon of the squid is close to the conductivity of sea-water.

It is also held that the $K^+$ ions are bound. Professor Ernst puts forward the following arguments to support this view: (1) Potassium ions are selectively concentrated by the muscle from solution. The process of $K^+$ concentration does not require energy. The maintenance of the $K^+$ concentration in the cell is not in the nature of a stationary-state effect ($K^+$ is retained by muscle at 0°C). (2) When muscle proteins are precipitated from a homogenisate, the greater part of the $K^+$ ions passes into the precipitate. (3) The concentration of $K^+$ in single muscle structures significantly exceeds the mean concentration for the whole muscle. (4) Measurement of the ionic concentration by a non-electrode method indicates an activity of the order of 0·5.

Undoubtedly, many of these views, and also the arguments in favour of the membrane theory, can be disputed or interpreted in other ways. However, it is possible that both these groups of facts are not mutually exclusive. The author proceeds from the idea that $K^+$ ions are bound in the cell and puts forward the hypothesis that the mechanism by which $K^+$ ions are accumulated in the muscle is similar to the process that occurs in ion-exchange resins. This view of the author deserves the most serious attention. It may be that this similarity is more than a simple analogy. Unfortunately,

we know little about the electrical properties of ion-exchangers and the nature of the bonds they form with ions.

In the second part the author pays great attention to questions relating to the reduction in muscle volume with excitation, an effect which he himself discovered as early as the 1920's. The author associates this effect with electrostriction arising from hydration of ions. This interpretation stems from the general concept of the author on the process of excitation and can be accepted only on presentation of unqualified proof of the re-formation of ions at the moment of excitation. There is still no direct evidence on this point, but the author considers that it is in fact proved (pp. 150–152).

The pages devoted to the analysis of the analogy between semi-conductors and muscles and nerves are of great interest. The author lists the following similarities: (1) one-way behaviour in synapses and nerve-muscle endings, (2) the necessity of a threshold value in order to evoke a response, (3) the development of a potential difference between the heated and cold parts of a living muscle, (4) a decrease in nerve resistance on heating, (5) the sensitivity of the muscle to damage and (6) restoration of muscle excitation by irradiation with ionizing radiation, besides a number of other, less important similarities.

The question raised by the author is of extraordinary importance for the whole problem of the structure of living matter. To our knowledge, up to now there has been no mention in the literature of a reliable proof that biological materials have semi-conducting properties. Unfortunately, the list of features in which semi-conductors resemble biological entities is based on purely external similarities; it does not follow that the processes are of the same nature. The question therefore remains open. The experimental tests which the author is carrying out and those he intends to carry out will be of great value.

The section devoted to changes observed in muscle with the development of tension is interesting. The author puts forward a number of arguments to prove that when tension develops and a muscle stretches, the structure assumes an ordered arrangement and crystallization of the muscle proteins occurs. This view is supported by the X-ray diagrams and by the volume decrease on contraction, which is independent of temperature.

Some problems of the thermodynamics of muscle contraction and muscle efficiency are discussed in the sections on "The Mechanical Aspect of Muscle Action" and "The Specific Function of Muscle in Metabolism". The author correctly points out that the efficiency of the working elements of a muscle cannot be evaluated by the widely accepted method of determining muscle efficiency based on effective working capacity and basal volume. Notwithstanding the opinion of Hill, the author considers that it is possible to differentiate that part of the energy in muscle which is directly associated with the contraction process and, the maximum power that the muscle can develop, and its associated heat production having been determined, to calculate the efficiency of the operating elements of muscle directly. The author bases this view on the difference in the nature of the temperature dependence between the fraction of the heat

VOL
5
196

produced in connection with the contraction process and that produced in connection with basal metabolism.

The last section of the book is devoted to general problems of the theory of muscle contraction. The author gives a very short survey of hypotheses that have been developed recently: the nature of the relation between the excitation process and contraction and the main contradictions of the membrane theory as applied to muscle are analysed. On chemical mechanics, the author touches on the extremely important question of the possibility of the conversion to chemical energy of the energy communicated to the contracted muscle during stretching.

This covers the main questions discussed by the author. Original experimental and theoretical treatment of many of these permits a new view to be taken of current hypotheses on muscle excitation and contraction and points out new experimental routes to their elucidation.

VOL. 5 1960

It should be mentioned that in addition to problems of muscle contraction the author deals with some questions of scientific ethics. He warns young research workers against dogmatic views and the danger of theories that conceal false premises behind experimental originality.

An absence of a conclusive and integral presentation of the author's views on the general nature of the processes developing during muscle excitation and contraction, and also some overloading with polemical matter — these are some of the defects of the book. The division of the book into sections and the assignment of chapter headings are not particularly well done. For example, the second part of the book which is mainly concerned with the physico-chemical properties of muscle is termed "Muscle Mechanics", a heading that does not reflect the contents of this section.

Undoubtedly, these defects, and some others not worth mentioning, in no way detract from the value of the book. The questions raised by the author are extremely topical and of interest to a wide range of research workers concerned with general problems of modern biology. The detailed analysis of the main problems of muscle contraction, the authors endeavour to reach solutions to them objectively and the clear division between experimental material and suggested hypotheses make this book a valuable contribution to the biophysics of muscle contraction.

B. N. VEPRINTSEV
*Translated by* E. HAYES

**D. N. NASONOV.** *Local Reaction and Diffuse Excitation of Protoplasm.**

This monograph by the famous Soviet cytologist and physiologist D. N. Nasonov appeared in 1959 and is concerned with fundamental problems of general and cellular physiology. As the title indicates the book is devoted mainly to the question of the interrelation of these two types of excitation.

* Biofizika **5**: No. 1, 105–106, 1960.

Drawing on a vast store of factual material amassed over a period of 30 years in various laboratories under his direction, and also on a wide and varied range of published data, Nasonov is able to develop his protein theory of excitation in full. Phenomena associated with the action of a variety of agents on living material are examined. By this means a whole series of changes can be demonstrated which are common to all living matter. Nasonov associates the universal features of these reactions with the protein nature of all living matter. This hypothesis is supported by an array of factual evidence proving the similarity of these phenomena with those which are observed in model experiments on isolated proteins subjected to denaturation.

The author's exposition of his protein (denaturation) theory of excitation is distinguished by a determination to establish the physicochemical bases of the changes occurring in protoplasm and to relate the physiological responses normally observed to their underlying causes. This is in full accord with the modern tendency to extend biological investigations down to the molecular level. The protein theory of excitation has proved fruitful both from a theoretical and a purely practical standpoint. For instance the author has devised a whole range of techniques whereby it is possible to determine the degree of alteration and excitation in tissues in which this is impossible by ordinary methods.

In considering denaturation processes as the basis of "cellular excitation", Nasonov approaches the problem from a histirical angle. In Nasonov's view, in the course of evolution local processes of protoplasmic repair developed into a higher form of cellular excitation — diffuse excitation. The precise and diverse experimental work, cited in this book, prove that reversible denaturation of cellular proteins is also responsible for diffuse excitation. The membrane theory of excitation and its consequences are critically examined by Nasonov in the light of his own results.

Criticism of the membrane theory is based predominantly on fundamental work on cell permeability. Although this does not demolish the substance of the membrane theory, nevertheless, it shows clearly the contradictions inherent in the membrane theory in its present form. The representation of protoplasm as a complex coacervate system, whose water is a poor solvent for many substances, is a better and more simple explanation of the phenomena associated with the passage in and out of the cell of a very diverse range of substances.

A large part of the monograph is rightly devoted to the author's theory of gradual excitation. This theory, which is extremely logical in conception and is backed up by excellent experimental work, bridges the gulf which exists between the phenomenon of the localized electrical response of an excited tissue to a stimulus and that of the travelling wave of excitation. Nasonov is able to demonstrate conclusively the fundamental unity of these two processes, and to indicate the mechanism of transition from one to the other, thus providing a means of understanding the self-regulation of the magnitude of the impulse of excitation which passes along a nerve fibre.

It is worthy of note that a number of the postulations of the theory of gradual excitation have found experimental proof in the more recent work of Nasonov's students.

VOL
5
196

It will be apparent from the above that all the questions examined in this monograph are logical developments of the idea of the unity of local and diffuse excitation, which in turn is based on the protein theory of excitation. This material is of undoubted value from a methodological standpoint.

This book is by no means a specialized work of limited appeal, despite the single-minded presentation of the material and its subordination to a fixed idea. A wide range of the most fundamental and exciting problems in present-day general physiology are examined in Nasonov's monograph (e.g. electrical tonus and accommodation, the amount of electricity and the energy of the stimulating current required to produce excitation, techniques of measuring excitability, etc.). Nasonov has been successful in combining theoretical postulations and conclusions with practical recommendations; the chapters devoted to discussion of the errors inherent in chronaximetry and to the elaboration of a new method of evaluating excitability can be cited as examples of this. This synthesis gives Nasonov's fundamental studies particular value. The appearance of this monograph is an outstanding event in the annals of Soviet physiological science.

VOL.
5
1960

V. N. CHERNIGOVSKII
*Translated by* C. PRINGLE

## SCIENCE NEWS

# ENERGY TRANSFER WITH SPECIAL REFERENCE TO BIOLOGICAL SYSTEMS*

(FARADAY SOCIETY DISCUSSION)

A GENERAL discussion on "Energy Transfer with Special Reference to Biological Systems" was held at Nottingham University on 14–16 April 1959. This international symposium was attended by 153 English and 55 participants from other countries (the U.S.S.R., U.S.A., Holland, Italy, Canada, Sweden, Belgium, Israel, German Democratic Republic, German Federal Republic, Switzerland and France). The main speakers invited by the organizers numbered 26; 10 from England, 12 from the U.S.A., 2 from the German Federal Republic and 1 from Holland; the U.S.S.R. was represented by the author.

The programme of the discussion comprised two sections: I. "Modes of Energy Transfer from Excited and Unstable Ionized States" (10 papers); II. "Energy Migration in Organized Biological Systems" (16 papers).

The first section was devoted to the transfer of excitation energy in liquid and solid solutions of fluorescent organic compounds and in luminescent organic crystals, excited by ultra-violet light or $\gamma$-radiation. The second section included lectures on the electrical

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conductivity of proteins, the photoconductivity of chlorophyll and haemin, triplet state reactions of chlorophyll in solutions, the state of chlorophyll and energy transfer in chloroplasts, transfer of excitation energy to haem in myoglobin, occurrence of energy migration in retinal rods the spectra of iron-derived porphyrins, the spectroscopy of respiratory enzymes and other topics.

In the present paper I shall give a detailed review only of the biological section of the discussion†+. This section was opened by Szent-Györgyi's introductory paper on the problem of energy transfer in biology. As distinct from his previous addresses and papers, the speaker on this occasion did not reduce the basis of bioenergetics to the presence and behaviour of the triplet state in living systems, as it was graphically represented by him in the book "Bioenergetics", published in 1956. Szent-Györgyi has now adopted a more acceptable position in considering that electron transfers, i.e. oxidation-reduction processes of the general type, are possible in biological systems between electron-donor and electron-acceptor components — the participants in biochemical reactions. For this purpose he presented a scale of the relative position of oxidation-reduction levels, basing his view on quantum-mechanical calculations, made by the French theoretist Pullman, of the relative energy levels of electron donor–acceptor relations for complex biologically important organic molecules Szent-Györgyi now regards energy migration in a biological structure more as the transfer of an electron- than the transfer of energy from the triplet state, i.e. he has returned in some measure to the semiconductor hypothesis which he put forward as long ago as 1941.

Szent-Györgyi's introductory paper was followed, in accordance with the pro gramme, by the paper of Prof. Eley and Cardew, on the theme "The Semiconductivity of Organic Substances — Part 3. Haemoglobin and Some Amino Acids", and the paper of A. N. Terenin, E. K. Putseiko and I. A. Akimov, on "Energy Transfer in Systems of Connected Organic Molecules".

In the Physical Chemistry Department of Nottingham University Professor Eley has been occupying himself for about 10 years with measurements of the electrical conductivity of proteins in order to check the validity of the semiconductor protein model. In this study he measured at constant potential (up to 120 V) the increase in conductivity of fine compressed layers of these substances in powder form when heated to 150°C. From the exponential increase of the conductivity the authors determined the values of the thermal activation energy required for the manifestation of conductivity in these almost non-conducting objects (resistance $10^{11}$–$10^{12}$ Ω). The following values (in eV) were obtained: 2·97 for globin, 2·75 for haemoglobin, 4·07 for β-alanine, 2·92 for glycine and 3·12 for polyglycine. The authors interpret these figures as the amount of energy required for the transfer of the valence electron to the conduction band, where it acquires mobility and can migrate through the system of hydrogen bonds.

† The reader can find a brief account of the whole discussion in the author's article in Vest. Akad. Nauk SSSR 8, 1959.

+ *Translator's note*. The full report of this Discussion has now been published in Discussions of the Faraday Society No. 27, 1959.

Confirmation for this last hypothesis was seen by the authors in the fact that the electrical conductivity of a single glycine crystal is a little higher in the direction parallel to the plane containing the chains of hydrogen bonds (C = 0 ... H-N), and also in the fact that the copper complex of glycine is non-conducting. The authors tried to determine the sign of the current-carrying charges by measuring, under great difficulties, the direction of the thermoelectric power, as is done for typical inorganic semiconductors, and found that for a glycine single crystal the thermoelectric power was n-type. The authors worked with specimens dried in vacuo and precluded the possibility of a purely electrolytic origin of the observed conductivity. To estimate the mobility of thermally liberated electrons in globin the authors employed the formulae used for intrinsic inorganic semiconductors and obtained an obviously unacceptably high value for the mobility, a value rarely observed even in the best inorganic semiconductors. This numeric discrepancy, and also the fact that artificial polyamides give approximately the same value of the thermal activation energy, led the author of this review to pose the question of how far the obtained activation energies of the order of 3 eV were characteristic of amino-acids and protein. Might they not be of the same origin as for any organic insulators, for instance cellulose or other biologically inert compounds. As distinct from protein, ferrihaem with a low activation energy of 1·74 eV, as also the synthetic dye phthalocyanine, fall into the category of typical organic semiconductors according to the studies made in our own country (A. T. Vartanian).

VOL. 5 1960

In my paper (co-authors E. K. Putseiko and I. A. Akimov) I gave an account of our investigations of electron liberation by light in layers of crystalline chlorophyll and its analogues — haemin and other tetrapyrrolic pigments. The investigations were carried out by measuring the resulting alternating potential, and this enabled us to determine also the sign of the current carrier. For the majority of these pigments we found that the photocurrent was of a positive nature. This means that the primarily liberated electron is held at first at the trapping level, while the electrons at the lower (ground) level of the pigment molecules become mobile, and migrate by a relay mechanism of intermolecular oxidation-reduction electron exchange. It followed from the spectrum of the crystal layers that the bands of the generalized conductivity levels in these pigments must be extremely narrow, if they exist at all, i.e. that the mobility of electrons in them must be extremely low. The experimental facts which we established must be taken into account in the semiconductor theory of chlorophyll action during photosynthesis, a theory which rests at present on assumptions not verified by experiment. In the second section of our paper I described the existence of transfer of excitation energy from a foreign dye to chlorophyll adsorbed with it on the surface of an inorganic semiconductor. Finally, in the last section of our paper we touched on the work carried out with the participation of A. V. Shablia, on the phenomenon of light-induced proton transfer in the intermolecular hydrogen bond C-O- ... H+N, an effect which can be observed from the change in the fluorescence spectrum.

In the discussion on our report Prof. Rabinowitch (U.S.A.) drew attention to the perfect correspondence between our observed spectral maximum of the photoelectric sensitivity of the chlorophyll layer with the maximum of the absorption spectrum of

a polycrystalline monolayer of this pigment, studied in his laboratory. Rosenberg (U.S.A.), referring to his experiments on the existence of photoconductivity in glass-like β-carotene, cast doubt on our thesis that only the crystalline form of chlorophyll and its analogues is photoconductive. Williams (England) pointed out that in our spectrum of the photoelectric sensitivity of the haemin layer there were maxima which could be compared with the transfer of an electron from the porphyrin skeleton to the central ion of trivalent iron in accordance with his interpretation of the absorption spectra of metal-containing porphyrins*. Weller (West Germany) referred to the contradiction between the direction of proton photo-transfer, as observed by us, and that which follows from his experiments on the variation of the proton-acceptor capacity of acridine on excitation by light in solutions. In reply to the last observation it was explained that in our experiments (solid frozen medium), as distinct from Weller's, proton transfer apparently did not take place at the excited level of the acridine molecule, but during the time when the molecule was in its ground state, excited intermittently by the emission of the fluorescence spectrum.

Mason's (University College, London) short paper entitled "Charge Transfer Processes in Biological Systems" consisted of the author's very general, controversial, concepts unsupported by experimental data of his own. It dealt with the mechanism of the effect of gases on the sensitivity of tissue to radioactive emission and with the nature of the carcinogenic action of aromatic hydrocarbons. Mason subscribed both to Szent-Györgyi's viewpoint of the important role of the presence of the triplet state in the protein system, and to the views about electronic conductivity in proteins. The latter requires a high level of excitation, of the order 76 kcal (3·3 eV), according to the theoretical calculation of Evans and Gergely (1949). The existence of non-localized energy bands was confirmed, in his view, by the experiments of Eley described above.

Mason suggested that carcinogenic derangement in the protein was associated with manifestation of electron mobility in it. To produce this mobility one of the electrons from the filled valence system of the protein must be removed, and this can be effected by radiation, or ultra-violet action when a valence band electron is raised to the vacant "conduction" band lying at a height of about 3·3 eV, as already stated. The author, however, foresaw another hypothetical possibility of the loss of an electron from the filled system of the valence levels of the protein. Below the outer filled valence band of levels, at a depth of 3·2 eV, according to the calculations of Evans and Gergely, the protein model has another, deeper, electron-filled ground band of levels. The author suggests that the molecule of the polycyclic aromatic hydrocarbon, attached to the protein, is capable of capturing an electron from the valence band of the protein, if among the vacant excited levels of the hydrocarbon there are those which exactly coincide in height with the valence system of levels. Here the author arbitrarily locates the lowest (ground) level of the hydrocarbon molecule at the same height as the ground band of levels of the protein.

* See account of Williams' paper in the review of the biochemical section of the discussion in the journal Biokhimiia 1, 1960.

VOL
5
196

Mason ascribes the specific carcinogenic action of particular polycyclic hydrocarbons to the coincidence of the 3·2 eV energy gap between the two indicated electron-filled energy bands in the model of the protein molecule and the 3·24 eV gap existing between the ground and vacant levels of aromatic polycyclic compounds of a particular structure Since the protein bands of electronic levels are narrow ($\pm$0·2 eV), the suggested criterion isolates six biologically active molecules out of forty hydrocarbons of similar structure, only one inactive molecule, anthanthrene, falls within this range. The speaker suggested that this proximity of the upper filled (valence) band in the semiconductor model of the protein molecule to the electronfree band of upper levels of the hydrocarbon molecule promotes the transfer of an electron from the protein to the hydrocarbon in the complex which they form together. In what way the manifested intramolecular mobility of the electrons should promote carcinogenic action the author did not explain, and confined himself to the assertion that after the loss of the electron the keto-enol regrouping along the polypeptide chain can easily take place, thus destroying the normal configuration. In passing, the author cited published information on the covalent attachment of the polycyclic hydrocarbon to the protein, thus contradicting his proposed concept of intermolecular charge transfer, but he considered this circumstance of no importance.

OL.
5
960

We should note, first of all, that a coincidence of the filled energy band of the protein with the vacant level of the hydrocarbon occurs not only for the first, lowest unoccupied level of the latter, but in most cases for higher — second or third — excitation levels.

Mason cited the sizes of the gap between two neighbouring excited levels for carcinogenic hydrocarbons, and he compared these values with the carcinogenic activity, on the assumption that the activity is due to the transfer of an electron, accepted from the protein, from the second or third excited level to the lower first excited level. In a series of hydrocarbons, from 3:4-benzophenanthrene to 3:4:8:9-dibenzpyrene this gap between the levels increases from 0·33 to 1·55 eV. The author does not stipulate what significance this value of the energy evolved in the indicated electron transfer between levels of the hydrocarbon anion* has for the mechanism of the process, and confines himself merely to a hint of the possibility of its propagation in the form of an "exciton" by the protein "lattice" and the "trapping" of this energy by nucleic acid with the breakdown of the hydrogen bonds in it.

The mechanism of carcinogenic action, as suggested by Mason, is very far-fetched, since it is not the differences between the energy levels which are important for intermolecular electron transfer, but their relative height in the scale of oxidation-reduction potentials, i.e. the energies of breakaway and attachment of the electron, values which are still unknown for the given system. Hence the location of the lower levels of the protein and hydrocarbon at the same height, from which the energy calculation is derived, appears arbitrary.

* Mason fails to consider that electron capture must lead to a change in the position of the levels in the hydrocarbon anion formed.

Even more speculative is the explanation of the marked increase in the sensitivity of tissues to radioactive emission in the presence of oxygen and nitric oxide, and also to the desensitizing action of inert gases in this process. In particular, Mason attributes the action of $O_2$ and NO to the purely physical effect of their paramagnetic molecules, which enhances, in his opinion, the formation of the triplet state in the protein, i.e. the breaking of the valence bonds of the $\pi$-electrons. The opposite effect of inert gases (helium, xenon, hydrogen, nitrogen and others), the author ascribes not to their displacement of oxygen from the tissues, but to their hypothetical deactivation of the "excited" electrons of the protein. In corroboration of his hypothesis the author finds a parallelism between the desensitizing effect of inert gases on protein radiosensitivity and the interaction of molecules of these gases with slow electrons in vacuum conditions, i.e. an effect having no direct relation at all to the effect in question. However, in a footnote the author renounces this arbitrary correlation of heterogeneous facts and refers merely to the greater or less degree of adsorption of molecules of inert gases on protein in relation to their polarizability and ionization potential.

The assumption of the author that the paramagnetic molecules of $O_2$ and NO are capable of forming a triplet state in the protein is erroneous. As we know from the results reported by Porter and Wright in the first section of the discussion these gases are exceptionally effective at "quenching", i.e. removing the triplet states of aromatic molecules. The author's reference to Evans' work for the appearance of a singlet-triplet absorption spectrum in the presence of $O_2$ and NO is based on a lack of understanding: the quantum considerations show that the triplet state half-life is shortened so that the transition from the ground (singlet) state on a triplet is facilitated.

This careful, critical analysis of Mason's short report was called forth by the fact that owing to its brevity and the vagueness of his formulations we could not bring out the essential points and evaluate their significance at the time.

Of great fundamental importance were the results communicated by the English biochemists Weber and Teale from the Department of Biochemistry of Sheffield University, in a paper entitled "Electronic Energy Transfer in Haem Proteins".

We know that compounds of CO with haem proteins (myoglobin, haemoglobin etc.) are decomposed not only by light absorbed directly by the haem, which is localized on the surface of the globin, but also very effectively by light absorbed by aromatic amino-acid residues distributed deep inside the globin molecule*. Conjugates of haemoglobin with foreign fluorescent dyes attached to its surface are also capable of splitting off CO under the action of light absorbed by the dye. These facts cannot be interpreted as the migration of a quantum of excitation energy through the protein structure, but can reasonably be explained by the inductive transfer of excitation energy to the haem from the fluorescent amino-acids or from the conjugated dye, without involving the structure of the protein carrier, which plays only the role of solvent. According to the well-known theory of Perrin, Vavilov or Förster, the electromagnetic inductive coupling and the transfer of excitation energy from the fluorescent

* See the review of Iu. A. Vladimirov and S. V. Konev in Biofizika **2**: No. 3, 1957.

molecule* take place on the condition that their mutual distance does not exceed 100 Å and the fluorescence spectrum of the energy donor overlaps the absorption spectrum of the acceptor. The latter, as in the case of haem for instance, may not even possess fluorescence. Hence, if the geometrical dimensions of the globin molecule provide this proximity of the haem with the light-excited donor centres (aromatic acid residues or conjugated dye molecules), then inductive transfer of excitation energy will take place when the requirement of spectral overlap is met. The studies of Weber and Teale were also devoted to an experimental demonstration of the fact that haem actively serves as an excitation acceptor, causing "quenching", i.e. a lowering of the fluorescence intensity of these donor centres.

Measurements of the yield of ultra-violet fluorescence of tryptophan residues in a number of native haem proteins (myoglobin, haemoglobin, catalase) showed that after removal of the haem the yield increased more than a hundredfold. Only in the case of peroxidase did the yield fail to increase much (7 times). The absolute quantum yield of fluorescence of haem-free globins was 0·2, whence it follows that 0·8 of the number of absorbed light quanta is missing as a result of radiationless processes. In the presence of haem the quantum yield of fluorescence falls to 0·002, which means that the probability of transfer of excitation energy to haem is 100 times greater than the probability of radiation and hence the probability of radiationless deactivation is certainly increased. In other words, the quantum yield of the transfer of excitation energy to haem must be considerable. This accounts for the high efficiency of the indirect photodecomposition of carboxyhaemoprotein by light absorbed at fluorescent centres. We must bear in mind that the quenching effect of haem described here, observed in the absence of CO, is due only to the removal of energy by the haem with its degradation into heat, without photodissociation taking place.

Weber and Teale also measured a similar quenching effect due to haem in conjugates of haem proteins with the dye dimethylamino napthalene sulphonyl chloride (DNA), which fluoresces under visible light. We cite the ratios of the fluorescence intensity $F_0$ of the conjugate after removal of the haem to the intensity $F$ before removal. These figures refer to a surface concentration of one dye molecule per haem protein molecule. When the number of dye molecules is raised to 3–4, $F_0/F$ diminishes.

| Haemoprotein | $F_0/F$ | R (Å) |
|---|---|---|
| Myoglobin $Fe^{2+}$ | 32 | 47 |
| Myoglobin $Fe^{3+}$ | 15 | 41 |
| Peroxidase $Fe^{2+}$ | 11 | 53 |
| Peroxidase $Fe^{3+}$ | 7 | 48 |
| Cytochrome *c* ($Fe^{2+}$, $Fe^{3+}$) | 100 | 30 |
| Haemoglobin $Fe^{2+}$ | 60 | 51 |
| Haemoglobin $Fe^{3+}$ | 40 | 47 |
| Catalase $Fe^{3+}$ | 7 | 52 |

* See the reviews: A. N. Terenin, Usp. fiz. nauk. **43**: 34, 1951; A. N. Terenin and V. L. Ermolaev, Usp. fiz. nauk. **58**: 37, 1956; Iu. A. Vladimirov and S. V. Konev, Biofizika **2**: 3, 1957.

It follows from these data that the removal of haem leads to a considerable increase in the fluorescence yield of the conjugate, and this is particularly significant in the case of cytochrome *c*, haemoglobin and myoglobin.

The last column contains the experimentally evaluated constant $R$, which characterizes, according to Förster's inductive transfer theory, the intermolecular distance at which the probability of quenching of the donor (as a result of transfer) equals the probability of the act of its emitting excitation energy in the form of a quantum of fluorescence. If the inductive transfer mechanism is valid in this case, then the cited constant $R$, calculated from these experiments, must, firstly, be commensurable with the size of the globin molecule and, secondly, correspond to the theoretical value of this constant evaluated, according to Forster, from the degree of overlap of the spectra of donor and acceptor, and from the lifetime of the excited state of the donor (in this case DNS). The latter value is known from other conjugates of this dye, which do not contain haem. The diameters of haem protein molecules are actually comparable with the values in the table, namely: myoglobin 34, haemoglobin 54·5, peroxidase 47, cytochrome *c* 16·5 and catalase 84 Å. The constant $R_{theor}$, calculated theoretically from the overlap of the fluorescence spectrum of DNS and the absorption spectrum of haem in myoglobin was equal to 58 Å, in good correspondence (considering the error involved in their determination from the experimental data) with the values 37–41 Å given in the table. The mathematical calculation of the experimental data, carried out on a computer, was based on a statistical treatment of the fluorescence of centres randomly distributed over the surface of the haemoprotein molecule and subject to inductive quenching by one haem (myoglobin) according to the law of inverse proportionality of the sixth power of the difference between them (to Förster's theory). From the calculations smooth graphs connecting the value of the quenching of the aggregate fluorescence with the value of the parameter $R$ were drawn. From the quenching value observed in the experiment it was possible in this way to obtain the experimental values of $R$ given in the table. In the case of four-haem proteins (haemoglobin and catalase) it cannot be assumed that the haems are concentrated at one place in the form of a fourfold "quencher" since this would require a non-uniform distribution of the fluorescent molecules on the surface of the globin. A satisfactory result is obtained from a model in which the four haems form two pairs at opposite ends of the diameter of a spherical molecule. Finally, it is very remarkable that the transformation of $Fe^{3+}$ into $Fe^{2+}$ in the haem of myoglobin is accompanied by a decrease (by a factor $1{\cdot}74 \pm 0{\cdot}24$) in the fluorescence intensity of its DNA conjugate. In this case the absorption spectrum of haem is shifted, and hence the degree of its overlap with the fluorescence spectrum is altered, increasing by a factor of 1·65 — in good agreement with the observed increase of "quenching".

The quantitative results obtained in this investigation leave no doubt at all of the inductive transfer of excitation energy of aromatic amino acids or of surface fluorescent molecules to haem, which emerges as an energy "trap".

In a very short communication, touching on the preceding topic, Professor Gibson of Sheffield University showed that the primary product of the photodecomposition

of carboxyhaemoglobin under the action of a powerful flash of light (wavelength 310 mμ) was not the usual haemoglobin but a short-lived, quickly-reacting product, of the radical type, reacting with CO more rapidly than the usual haemoglobin. The rate of its reaction with CO was 40 times greater than the usual haemoglobin and the energy of activation of the reaction was lower (5·6 kcal instead of 10·5 kcal). This active intermediate product spontaneously and rapidly changed into the usual haemoglobin with a rate constant of 250 $sec^{-1}$ (at 0°). The author considered this intermediate product as an unstable haemoglobin molecule which has lost in the first instance only one CO molecule and which has not succeeded in acquiring the equilibrium protein configuration, at which the succeeding ones are split off. The author pictured the primary removal of CO as a kind of release mechanism for the rapid reconstruction of the bonds extending through the protein part of the molecule.

Excellent technique was shown in the interesting investigation carried out by Hagins and Jennings. The theme of their paper was "Radiationless Migration of Electronic Excitation in Retinal Rods". The high physiological efficiency of quanta of green light absorbed by rhodopsin molecules at pigment concentrations exceeding $10^{-3}$ M in the rods of the retina naturally leads to the hypothesis that there occurs there the same inductive energy migration, which was the subject of the preceding papers. The work of the authors was devoted to the experimental verification of this hypothesis. If we prepare an artificial gel containing randomly oriented, isolated, dark-adapted outer segments, of rabbit retinal rods, then this system shows marked dichroism after the partial bleaching of the rhodopsin by polarized light. That is, on examination of the gel layer in monochromatic polarized light (486 mμ) with the electric vector parallel to that which initially caused the bleaching of the rhodopsin, the transmission of the layer will be greater than in the case where the light vector on examination is perpendicular to the one which acted. This well-known photodichroic effect is attributed to the fact that the bleaching mainly affects those anisotropic pigment molecules in which the axes of greatest absorption are parallel to the electric vector of the incident light. For measurement of the dichroism manifested on illumination the authors employed an extremely sensitive differential photoelectric arrangement giving a continuous trace of the phenomenon in time, and this enabled them to detect the slight difference in light transmission in two mutually perpendicular orientations of the polarizer before and after illumination with brief pulses (20 μsec each) over a period of 50 msec. It was found that, in sharp contrast to the gel containing isolated rods, no photodichroism was manifested in the undamaged retina of the rabbit, although the rhodopsin molecules in the rod segments are known to be fixed in particular planes, and hence on even more marked photodichroism was to be expected than in the artificial gel. The explanation of this striking fact might be sought in the inductive intermolecular transfer of excitation energy, where the energy migrates from the rhodopsin molecules initially excited by polarized light to those oriented perpendicularly to them, and thus causes the bleaching of the molecules which were not directly affected by the light.

For a quantitative assessment of the possibility of this mechanism the authors made measurements of the concentration depolarization of fluorescence not for rhodopsin,

the fluorescence of which is very weak, but for its analogue — vitamin A in solution. From these experiments, and also from measurements of the overlap of the absorption and fluorescence spectra (see the paper by Weber and Teale) it was found that this chromophore manifests insignificant inductive interaction in the excited state at these intermolecular distances (55–112 Å), which must be the case for rhodopsin in the rods.

Finally, the authors performed an elegant experiment on the excitation of rhodopsin in a single rod, by a very fine light probe (width 0·5 μ) and observed under a microscope if the bleaching extended beyond the limits of the probe. They found that the excitation and bleaching did not extend beyond the limits of the probe.

Thus, the whole set of measurements led to the conclusion that inductive migration of electronic excitation energy between rhodopsin molecules has little or, rather, no significance at all for the physiological mechanism of excitation in the rods. The absence of photodichroism in the undamaged retina is wholly explained, in the author's opinion, by the Brownian rotation of the pigment molecules.

VOL
5
196

The authors conducted the different parts of their work in three institutions, two of them medical institutions in the U.S.A. — the Laboratory of Physical Biology of the National Institute of Arthritis and Metabolic Diseases (National Institute of Health) and the Naval Medical Research Institute, and in the Physiological Laboratory of Cambridge University (England). This method of working, where two or three investigators from different institutes in different towns or even countries collect in one laboratory which offers the best facilities, is practised fairly often, judging from a number of the papers.

Less clear-cut, though interesting, results were given in the paper of Johnson, Eyring and Chang "Reaction Rate Control of Light Emission in Bioluminescent Systems". These authors were also representatives of different institutions in the U.S.A. — the Biology Departments of Princeton and Brown (Rhode Island) Universities, and the Department of Chemistry of the University of Utah.

The paper examined very simplified oxidation reactions (dehydrogenation by oxygen), including dark as well as light-producing reactions, for three kinds of organism: (1) a crustacean (*Cypridina*), which excretes a luminescent mixture of substances into the water, (2) bacteria and (3) fireflies.

It is well known that bioluminescence arises in the oxidation of a substrate (luciferin) catalysed by a heat-labile enzyme (luciferase). Luciferin and luciferase have been isolated and luminescence has been produced outside the organism, but only in the limits of the same species. However, success has recently been achieved in effecting the luminescent reaction between luciferin-luciferase components of different species of organisms, from a fish and a crustacean. Since it has been established that bacterial luminescence is associated with the activity of the flavine system the authors believe that all cells are potentially bioluminescent. In the case of *Cypridina*, the simplest bioluminescent system, the luminescence carrier is the oxidation substrate — luciferin, which was obtained in a crystalline state with a luminescent activity 40,000 times greater than that observed in the original dried organism.

Discussing the successive stages of proton transfer in dark and luminescent oxidation pathways (in aerobic respiration of bacteria), the authors arrived at a simplified scheme of stages of energy liberation and indicated, provisionally, the stages which are acted on by inhibitors of the ferricyanide or narcotic type (urethane or alcohol).

The main content of the paper consists in a kinetic analysis and the deduction of an equation for the luminescence intensity, according to the following simple scheme.

$$\underset{\text{(reduced diphosphopyridine nucleotide)}}{\text{A (DPNH)}} \xrightarrow{k_1} \text{B} \begin{cases} \xrightarrow{k_2} \text{products + luminescence (flavine system)} \\ \xrightarrow{k_3} \text{products without light} \end{cases}$$

where $k_1$, $k_2$, $k_3$ are rate constants of the reactions indicated by the arrows. The rather complex, very general expression which these authors obtained for the luminescence intensity was compared with the results of their experiments on the effect of high hydrostatic pressure (up to 6000 lb/in$^2$) on bacterial luminescence at different temperatures. According to the continuous recorded curves of the time course of the luminescence intensity a sudden application of pressure led to a sharp short flash, which rapidly decayed. When the pressure was removed there was a sudden brief quenching of the luminescence, which then gradually increased to its initial value. The course of the curves and the size of the flashes were different for different temperatures. From an examination of the rather complex curves obtained the authors concluded that pressure counteracts inhibitors (narcotics) present in luminescent bacteria. Experiments carried out on live tadpoles showed a similar action of high hydrostatic pressure in removing the effect of a narcotic (alcohol), this process being reversible. In addition, an experiment on the excitability of isolated nerve fibre also revealed that the narcotizing effect of alcohol could be removed by the application of high pressure (7000 lb/in$^2$).

This paper was obviously outside the topic of the discussion.

Apart from the above-mentioned paper of the author (jointly with A. I. Putseiko and I. A. Akimov) on the photoconductivity of chlorophyll, the problem of energy transfer in chlorophyll during synthesis was dealt with in the papers of Profs. Livingston and Pugh (University of Minnesota), "The Role of the Triplet State in Reactions Sensitized by Chlorophyll"; Prof. Lumry, Mayne and Spikes (Universities of Minnesota and Utah), "Fluorescence Yield against Velocity Relationships in the Hill Reaction of Chloroplast Fragments"; Prof. Rabinowitch and Brody (University of Illinois), "Primary Photochemical and Photophysical Processes in Photosynthesis"; Amesz and Duysens (Leiden University), "Spectrophotometric Studies on Pyridine Nucleotide in Photosynthetic Cells and Cellular Material".

These papers (apart from the last) contained discussion and experimental results in support of the view of migration of chlorophyll excitation energy within a limited system of about 200 chlorophyll molecules, forming a condensed non-crystalline phase (possibly an amorphous monolayer of chlorophyll molecules on an enzyme macromo-

lecule). (A detailed examination of the content of these papers is given in the journal Biokhimiia 1, 1960).

A great proportion of the remaining papers was devoted to various specific questions, of interest in themselves, but not containing new data of fundamental significance. A noteworthy feature was the excellent theoretical treatment of the results in the paper of Weber and Teale, who used the fluorescence method to demonstrate transfer of excitation energy from tryptophan or foreign fluorescent molecules to haem in heam proteins. The paper of Hagins and Jennings was very impressive. With convincing, ingenious experiments they demonstrated that there was no migration of energy between rhodopsin molecules in the visual process. This certainly implies a revision of the widely held view of the universality of energy migration in biological systems in cases where no convincing experiments are available.

A. N. TERENIN

*Translated by* F. L. SINCLAIR

VOL
5
196

## ELECTROPHYSIOLOGY AND BIOPHYSICS OF THE SENSORY ORGANS AT THE XXI INTERNATIONAL CONGRESS OF PHYSIOLOGISTS*

THE twenty-first International Congress of Physiologists took place in Buenos Aires from 9 to 15 august 1959. The first day of the proceedings was dedicated to the opening of the congress. During the subsequent days lectures were delivered and discussions took place on approximately eight hundred papers on various questions in the spheres of physiology, biochemistry, biophysics and pharmacology, arranged in 75 sections, 12 symposia and 16 conferences.

To fulfil such a large programme, 14 sections, 3 conferences and 3 symposia were arranged daily; 9–10 lectures were delivered and discussed at each sectional meeting, one at each conference and 2–4 papers were read at the symposia.

At the sectional meetings results of experimental studies were mainly reported; at the conferences and symposia a summary of certain problems was given. In addition, 6–7 films were shown twice daily and two exhibitions were organised — one demonstrated new apparatus, and the other illustrated the results of scientific achievements. Special demonstrations in the auditoria were also given.

Soviet physiologists delivered more than 30 lectures. As at previous congresses, the badge of a delegate, designating the country represented by the scientist, ensured a warm welcome for the Soviet physiologists from the majority of participants at the congress. The Soviet delegation provoked great interest in the Argentine public and particularly amongst students of the University, where the sessions of the congress

* Biofizika **6**: No. 1, 114–119, 1960.

took place. Crowds of young people surrounded us, constantly enquiring, with curiosity and excitement, about living conditions in the Soviet Union.

The authority of Soviet science has grown greatly in recent years; this was clearly demonstrated at the congress by the election of two Soviet scientists, E. A. Asratian and S. V. Anichkov, as Vice-Presidents of the Universal Association of Physiologists.

Physiology and biophysics of the sensory organs were represented by a relatively small number of papers, namely 12 on the physiology of vision, and 9 on the physiology of the other sensory organs. Owing to the absence of several lecturers, only 17 lectures were delivered and one was given at a special conference.

Briefly, the majority of lectures were concerned with various questions on the physiology of systems of analysers and only one problem, that of the functional and morphological organization, together with the biophysics of the retina, was submitted in five communications and subjected to a detailed discussion. In order to acquaint readers with the total volume of work relating to the sensory organs, a brief review of those papers which have already been published in foreign literature is set forth below. The members of the conference heard a lecture delivered by one of the most outstanding specialists on electrophysiology of the retina, namely the Japanese scientist T. Tomita. At the beginning of his address Tomita dwelt on the results of his previous scientific achievements regarding the site of origin of the potentials in the retina. He established that the greater part of the fast components of the electroretinogram (R II and R III potentials) originate near the receptors of the retina, whereas the origin of the slow component (R I) is of much deeper localisation in relation to the receptors. Tomita demonstrated the presence of a slow, negative potential which can only be registered within the retina. This potential is of a localised character and is limited to the illuminated area of the retina. The impulse activity of the ganglion cells is firmly related to this local negative response.

Almost all results of the experiments enumerated by Tomita, with the exception of the first few, were subsequently confirmed by other research workers: Brown and Wiesel (1958), Brindley (1956, 1958), Guras (1958). Opinions regarding the site of origin of the speedy e.r.g. components are, however, divided: Ottoson and svaetichin (1953) and also Brindley consider that R II and R III potentials originate within the receptors; Brown and Wiesel localise the R III potential in the distal section of the receptors and R II in the structures which surround the outer plexiform layers.

According to Tomita, these controversies arose because of the difficulty in accurate assessment of depth necessary for the correct e.r.g. registration, since the microelectrode is liable to be bent when inserted into the retina. The lecturer dwelt on the methods perfected by him, namely, a pencil microelectrode, which consisted of two pipettes inserted one inside the other. The outer pipette, 2 cm in length and 5–15 mm in diameter, was filled with Ringer's solution. The very thin inner pipette, filled with 3 M KCL solution, could be freely moved in and out of the outer pipette. The second method consisted of *intra vitam* staining of the place of insertion of the microelectrode. The inner micropipette was filled with 10% potassium ferrocyanide solution, and following the registration of a definite depth in the retina, the ferrocyanide ions were

inculcated, by electrophoresis, into the tissues of the retina. A small dose of 1–2% ferric chloride solution was applied simultaneously to the retina, this gave rise to the formation of a blue spot in the tissues surrounding the pipette. Tomita recommended inserting the microelectrode from the side of the receptors, as the staining then takes place three times sooner.

The lecturer then proceeded to give a detailed account of the latest experiments carried out by him with the aid of the most recent modern methods. The results of these experiments confirmed his previous conclusion, namely, that the primary site of origin of the speedy R II and R III potentials is located nearer to the receptors of the retina.

By gradual insertion, into the retina, of the inner pipette emerging from the outer pipette which was placed at the distal surface of the outer, rods and cones, layer the e.r.g. reached half its value close to the proximal border of the inner nuclear layer. Tomita considered this place to be the origin of the e.r.g. fast components. He achieved the same results by *intra vitam* staining of the site of the microelectrode.

Tomita also determined the site of the R III potential by the following method: He took an e.r.g. from a zone of the retina, after removal of the outer layer of rods and cones. It transpired that this zone, deprived of the photo-sensitive cells, responded to light with a prolonged potential with a positive charge on the side of the receptors, whereas the normal uninjured portion of the retina gave a normal e.r.g. tracing. It follows, therefore, that the R III potential was isolated in the removed strip of the retina.

Another question arose concerning the localisation of the R-membrane in the eye of a frog. For this purpose use was made of a current of rectangular impulses (15 μA for 30 msec) passing through the retina. With the aid of pencil microelectrodes Tomita recorded the potential difference between the outer pipette, placed on the anterior surface of the retina, and the inner pipette inserted at varied depths into the retina. From the changes in the potential he calculated the resistance of the tissues between the pipettes. The responses of the eye to stimulus by the current and by illumination were registered alternatively.

The results of these experiments coincided with Brindley's findings — a sudden significant increase of the potential recorded at a depth of approximately 250 μ — which testified to the presence of an R-membrane.

Tomita, however, resolutely repudiated Brindley's point of view, namely that the R-membrane appeared to be the outer membrane of the retina, but confirmed that it is located precisely behind the retina. He proved it by the fact that the inner surface of the eye cup, deprived of the retina altogether, but with the pigmented epithelium remaining, had a resistance comparable with that of the R-membrane. After removing the pigmented epithelium the resistance disappeared completely.

Tomita assumed that the R II component was part of the intraretinal negative response and that the R-membrane was responsible for rendering the distal part of the retina more negative to the indifferent electrode.

The last question touched upon by Tomita in his lecture was concerned with the intraretinal response in the eye of a fish. He suggested the term "potential of cone

activity" should be replaced by the term "S-potential" as long as this response, discovered by Svaetichin, turned out to be of more proximal origin. Following the recent work in this sphere by Mc. Nicol and Svaetichin (1958), Tomita considered it essential to dwell on two questions only: first, whether the S-potential is of intra or extracellular origin. According to his experiments the S-potential is undoubtedly of extracellular origin because, with a distance of less than 30 μ between pipettes of the pencil-microelectrode, both pipettes registered identical S potentials simultaneously; the S potential registered by one pipette was reduced under the influence of currents applied to the other pipette. Secondly, the question regarding a connexion between the S potential and the impulse activity of the ganglion cells was considered. Experiments by Tomita have demonstrated that the S potential originates at the same depth as the fast e.r.g. component, i.e. around the proximal surface of the inner nuclear layer. When the pipette was moved forward, up to 50–60 μ, isolated impulses were frequently recorded.

VOL. 5 1960

Tests with white light showed either "on" or "off" discharges, whereas the S potential, in response to white light, was always negative. Consequently, notwithstanding the topographic proximity, these two types of retinal activity are interconnected only indirectly.

The lecture delivered by Tomita provoked lively discussion. Of particular interest were the ideas concerning the characteristic potentials originating from pressure of the microelectrodes at various levels in the retina.

Of equal interest was the intricate work by Svaetichin, G. and Krattenmaeher, W. (Venezuela), on "Photostimulation of Solitary Cone". While working with retinae from several fish species with large cones (10–13 μ in diameter), these authors observed the presence of separate receptors. By illuminating them with light passed through a diaphragm and projected, in the form of a circle, 5–8 μ in diameter, upon a solitary cone, the authors succeeded in obtaining stimulation of a solitary element owing to the total light reflection within the cone. In their opinion, the electrical response originated not in the cones, but in the star-shaped horizontal cells. All cones had the same threshold. The photopic response provoked by stimulation of a solitary cone was graded — its amplitude was approximately proportional to the log of the intensity of the stimulus. When a solitary cone was illuminated within a radius of about 50 μ from the recording microelectrode, the response was of maximal amplitude (300 mV); when, however, the luminous spot was moved further from the microelectrode, zero response was reached at a radius of 200–400 μ. When the luminous spot was widened and the light intensity remained the same, a linear increase in the amplitude of the electric response was shown, in proportion to the number of cones illuminated, as long as the illuminated zone did not exceed 70 μ approximately. Under such conditions the area of the luminous spot corresponded to the size of one horizontal cell and of approximately 100 cones, each provided with one synaptic terminal. Consequently, the action of cone synapses upon one horizontal cell was an additional variable in relation to the amplitude of the provoked responses.

From the same laboratory a paper was presented by Villegas, G. M. and Suter, J. (Venezuela), concerning "The Functional Organisation and Ultrastructure of the Retina

in Vertebrata", which was illustrated by microphotographs of the retina. With powerful magnification, not only all details in the structure, up to the limit of 0·01 μ, of the individual cells of the retina, but also all the finest remifications and outgrowths of various cell formations in the retina of vertebrata could be seen. The authors demonstrated the presence of a very complicated structure, 2–3 μ in thickness, in the retina of all the animals studied (fish, tortoise, frog and monkey), this is located between the synaptic endings of the receptor and bipolar cells and between the horizontal and amacrine cells.

This structure consists of an endless number of cellular outgrowths which form a three-dimensional network, the processes of which penetrate the projecting synaptic endings of the receptors. A similar structure was also seen in the foveal zone of monkeys. Three types of nuclei are to be found in the bipolar cells of the retina in primates and only two types in the fovea centralis.

Morphological investigations confirmed the likelihood of stimulation passing from one cell to another in the layer of the horizontal cells in certain fish species.

Also of interest were the Author's observations that the interlaminar spaces between the cones are equal to the diameters of the outer cone segments in certain fish that possess the faculty of colour discrimination. This observation refutes the physical theory of colour vision which is based upon various scales of light perceptors.

A further two papers were taken up with investigations on the character of responses from the ganglion cells of the retina. With the aid of micropipettes, Wiesel, T. N. (U.S.A.) made intracellular activity recordings from ganglion cells in the cat's retina. The resting potential (50–70 mV) was very constant in these cells. The character of impulses in the ganglion cells was similar to that derived from an extracellular electrode. Illumination of stimulated or inhibited areas of the receptive fields influenced the potential of the membrane and the frequency of the impulses. Stimulation gave rise to slow depolarisation of the membrane potential and to increased impulse frequency. This effect was intensified by stronger illumination. Illumination of the inhibited area of the receptive field produced the opposite effect.

From his experiments, the author concluded that the influences of stimulation and inhibition could provoke slow changes of the generator type in the potential of the membrane, which, in its turn, could determine the frequency of disharges in the fibres of the optical nerve.

Wagner, H. G. and McNichol, E. E. (U.S.A.) studied with the aid of metallic electrodes the activity in a ganglion cell isolated from the retina of a carp. The character of responses depended upon the wave length, intensity, and spatial correlation of the illumination with the receptor surface of the ganglion cell. Differences were observed in response from various ganglion cells. In some of them, the character of response changed from a predominantly active one during the period of illumination with short wave-lengths (less than 500 mμ), to a similar response after cessation of illumination with long wave-lengths (more than 600 mμ). Waves of intermediate length provoked potentials both during and after illumination.

These five lectures on the functional and morphological organisation of the retina should be regarded as a further stage of development in the field of such investigations. The lectures are of interest because of the exclusive, detailed exploitation of the methods employed in the experiments which gave the authors the opportunity of studying the finest mechanisms of response, arising from the first link of the optical system, to light stimulation.

A few communications were dedicated to the study of the functional organisation of the higher levels of the optic analysor.

The paper by Hubel, D. H. and Wiesel, T. N. (U.S.A.) provoked great interest mixed with some confusion. These authors studied the organisation of the receptor field of one solitary element in the striated cortex of a cat under light narcosis. The delicately adjusted retina was stimulated by restricted light. The arrangement of the receptor field was, in certain respects, identical, in the majority of the cortical cells, to that of the ganglion cells of the retina. Illumination of certain sections of the retina increased the activity of the cortical cells, whereas illumination of other parts inhibited it. Reciprocity was observed between various sections. When both types of sections were illuminated, a tendency towards extinction of the effect was observed. According to the authors' data, intensive stimulation covering both types of sections did not usually provoke any response at all. The authors failed to demonstrate in the striated cortex the concentric fields described previously for the ganglion cells. Stimulation and inhibition areas of the retina are represented in the cortex by specialised complicated patterns of diverse types. Thus, for instance, certain fields include wide and nanarrow stimulation areas with inhibition sections on both sides. Other fields show the opposite arrangement. The inhibition sections on each side are usually asymmetrical. A field of such a type can be orientated vertically, horizontally or at an angle.

The authors ascertained its orientation by exploiting the effect of a beam of light shining through a diaphragm that could be orientated in different directions. To-and-fro movements of the illuminated diaphragm were helpful in assessing responses to the movements of a stimulating agent. When the diaphragm was directed parallel to the field axis the maximum response was obtained.

Many fields were activated by binocular stimulation. A chart drawn for the receptor fields of each eye resembled those in the cortex itself.

This lecture provoked lively discussion. It is noteworthy that one can hardly be in agreement with the authors' interpretation of their experimental results-achieved from a standpoint of fixed regulation of stimulation and inhibition processes in the striated cortex — since such a standpoint is refuted by numerous experimental facts in favour of a mobile reciprocal action of stimulation and inhibition processes in the central nervous system.

Of curious interest was the paper by Marg, E., Hamasaki, D. and Giolli, R. (U.S.A.), in which, for the first time, a study was made of electrophysiological responses of the posterior accessory optical tract to light stimulation. The authors demonstrated that light stimulation of both contralateral and ipsolateral retina and electrical stimula-

VOL. 5 1960

tion of the optic nerves or of the chiasma provoked a response from the nucleus of the posterior accessory optic tract. At the same time, electrical stimulation of the nucleus itself did not provoke a response from the optic nerve. It follows therefore, that the nucleus possesses a centripetal, not a centrifugal, function. Experiments have shown that a synapse exists between chiasma and the nucleus.

Such papers defined more accurately our ideas about the physiological role of individual formations in the central nervous system. A lecture delivered by Pasik, T., Pasik, P., Battersby, W. S. and Bender, M. V. (U.S.A.) followed the same course. Experiments on monkeys gave the authors the opportunity of studying visual and tactile discernments, prior to and following bitemporal and biparietal section of the cortex. It was revealed that bitemporal destruction of the cortex interfered with the correct solution of visual problems, in particular with seeing small objects, while parietal damage did not cause such disturbances. The results of these experiments showed that bitemporal damage caused specific disturbances of vision. It changed the field of vision and was responsible for the inability to inhibit incorrect responses. With training, however, disturbances of visual discernment could be compensated.

Battersby, W. S. and Wagman, J. H. (U.S.A.) made a study of time and space parameters of visual stimulation, defined by a psychophysiological method on human beings and by an electrophysiological method on cats. Analysis of potentials, provoked in deeply anaesthetised animals, did not show any correlation between the results of investigations of psychophysiological and electrophysiological methods. With light narcosis, however, partial correlation between effects of time, but not of space was observed. Investigations by the authors demonstrated that both peripheral and central nerve processes play their part when visual sensitivity is assessed.

A paper by Palestini M., Armengol V. and Zeballos, G. (Chile) was concerned with a study of spontaneous and evocated rhythmic activity of the optic tract in anaesthetised cats. For this purpose, the authors made use of a stereotaxic method combined with the recording of potentials on an oscilloscope and with e.e.g. tracings. There types of potentials were recorded: (1) provoked potentials, 2 or 3 at a time with a latent period of 20 msec and with an amplitude of 100–500 mV; (2) sinusoidal activity following the provoked potentials with a frequency of 40–80 sec, lasting 200–300 msec and with an amplitude of 100 mV. With an interval of one stimulus per second or less between flashes the first stimulus only provoked sinusoidal activity; (3) during dark adaptation a constant rhythm with a frequency of 20–30 sec was recorded, which was instantly blocked by light.

One lecture was delivered in this section on the mechanism of pupillary action by Stark, L. (U.S.A.). His investigations demonstrated that pupillary servo-mechanisms must have an insignificant effect in maintaining a steady state. The logarithm of the diameter lags behind, threefold, an alteration of the light intensity. This is explained by the sluggish movement of the muscles of the iris. It was established that a significant number of murmurs is present in the pupillary system, which are transmitted from the other parts of the brain.

An analogue computer, arranged as a binary counter, gave the opportunity of elucidating the reciprocal action observed between experimentally encountered abnormalities.

A lecture by Benetato, A. (Rumania) concerned the role of ergotoxine and adrenaline in the regulation mechanism of intra-ocular pressure. The important role of adrenaline and of the functional state of the sympathetic nervous system in intra-ocular pressure was brought to light.

Two papers were read at the sessions of the section on the physiology of hearing. One, by Davis, H., Detatherage, B. H., Eldridge, D. H. and Teas, D. C. (U.S.A.), concerned a most detailed investigation of impulses relating to the auditory nerve. It was shown that the highly strained nasal consonant sound $N_1$ is proportionate, approximately, to the total number of action nerve fibres. Initial responses to brief stimuli are deprived of the effects of refractivity and adaptation. With sounds of 7000 c/s and duration of the ascending phase of 1 msec, consonant sound $N_1$ increases in intensity and reaches a height approximately equal to the force of sound pressure. The initial increase in intensity from the threshold is rather abrupt. A curve is generated from three aggregates of neurones which respond to various levels of intensity, although they overlap each other. The most sensitive aggregate of neurones innervates the outer hair cells of the basal spiral in the organ of Corti; the second part, the inner and outer hair cells; the third, the most apical area. The latter is stimulated by complicated cochlea responses which are provoked by high sound intensities only. Notes of 500 c/s and lower provoke a salvo of $N_1$ impulses for each sound wave. Synchronous impulses originate from the basal spiral, but those from the third and fourth spirals of the cochlea are delayed and desynchronised by a wave arising from movement of the basillary membrane and by individual oscillations during the latent interval. These impulses, being left behind, pool together with salvo $N_1$ from the basal spiral, which appears in response to the subsequent sound wave, although not by the simple route. Storing the impulses separately proved to be unsuccessful. The general entrance–exit curve resembles a power function with an index equal to —2 and —3. Chocholle, R. (France) investigated, on human beings, the effect of contralateral sounds of the same frequency for the purpose of demonstrating the differential threshold and the significance in the discernment of the interaural phases. The lecturer established that the differences in thresholds for 100 and 400 c/s are considerably higher when a constant sound of the same frequency is transmitted to the contralateral ear.

One of the most interesting lectures on sense organs delivered at the section for physiology was dedicated to the electrophysiological characteristics of the olfactory receptors by Beidler, L. M. (U.S.A.). Response to odour stimulation was recorded simultaneously from one branch of the olfactory and trigeminal nerves, which innervates the olfactory area in rats. The response of the olfactory nerve to weak odours did not alter from one inhalation to another, whereas in high odour concentrations an alteration in the subsequent responses was observed. The trigeminal response appeared in average odour concentrations and became stronger in high concentrations. The magnitude of response depended on type of odour, its concentration, velocity

of air movement along the epithelium, duration of stimulation and also on the level of sympathetic discharges in the nasal zone. Increased sympathetic activity, provoked by loud noise, eye illumination or by direct electrical stimulation of the cervical sympathetic nerve, significantly augmented the magnitude of olfactory responses to odours. The author reported the trigeminal and olfactory responses to sudden intensification or sharp weakening of the odour stimulant, to cessation of heart beat, to the degree of odour concentration etc.

Two lectures were delivered on skin receptors. Vendrik, A. J. H. and Eijkman E. (Holland) investigated adaptation of heat receptors, stimulated by short wave radiation, together with that of tactile receptors, stimulated by rectangular deformations of varied duration and by sinusoidal deformations of diverse frequency. It was established that, in addition to peripheral adaptation, the receptors show, simultaneously, signs of central adaptation, which can also be achieved by the direct route.

Douglas, W. W., Ritchie, J. M. and Staur, R. W. (U.S.A.) have demonstrated that a large group of non-myelinised fibres of saphenous nerve in cat, while responding to touch with discharges, respond also to cooling of the skin in a similar manner. The method used by the authors for demonstration of this effect is of interest: intricate action currents were provoked in the nerve trunk in such a manner that they showed an antidromic spread, past a pair of recording electrodes, for a considerable distance along the branch of the nerve. In a group of afferent fibres ortodromic sensory stimulations were revealed by observing the reduction of the amplitude in the correspondingly provoked heightened intricate action currents as result of a clash between antidromic and ortodromic impulses. Such original methods gave the authors the opportunity of demonstrating that the basic part of non-myelinised fibres in the saphenous nerve is sensitive to both types of stimulation.

From the brief account of the lectures delivered at the International Congress it is obvious that the investigations on the physiology of sense organs take a varied course, but the principal research carried out consisted, as before, in the most detailed electrophysiological studies of the characteristics of electrical activity, both of peripheral and of much higher levels of the analysor systems.

Scientific works by Tomita, Svaetichin and their collaborators on the potentials of the individual elements of the retina, work by Davis, in which the role of separate neurone groups in the conduction of sound stimulations was discovered, work by Beidler on the significance of olfactory and trigeminal neurones in the sense of smell — all this work was carried out with the highest degree of methodical perfection. Investigations of this kind progress only in our midst in the Union and it seems to me that physiologists in our country, working on the physiology of the sense organs, should not only master all the methods previously mentioned, but should also endeavour to display original investigations of such nature.

Side by side with these most interesting lectures, some of the work presented at the sectional sessions was of negligible interest. Some foreign research workers on the physiology of vision (Wisel and Hubel) together with many neurophysiologists advance theories concerning the presence of fixation points of stimulated and inhibited

processes in both the peripheral and the central substrata of the analysor systems. Such a conception merits no further discussion, since the total vast experimental work in Pavlov's laboratories and the experiments of the Soviet scientists on the physiology of the sense organs prove convincingly the indissolubility of existing nerve processes which act in an uninterrupted and reciprocal manner and which are located in the same morphological structures of the analysos systems.

Comparison of the contents of papers on physiology of sense organs delivered at the last two International Congresses of Physiologists (1956 and 1959) shows that no particularly significant discovery in this field of science has been made during the past three years by foreign scientists.

Progress has been made, however, in extending this knowledge and in perfecting these fundamental theories which, in the course of the last two decades, have been brilliantly demonstrated experimentally due to the astounding progress in the methods of investigation of electrophysiology.

VOL.
5
1960

It is necessary to underline that I have made this statement on the basis of the content of the scientific lectures and not on their number. The drastic reduction in the number of lectures delivered at the XXI Congress in comparison with the number given at the XX Congress (approximately 4 times less) is explained not by lowering of the scientific front amongst foreign scientists during the past 3 years in the field of physiology, but apparently by the great distance of the majority of eminent scientific centres from the Argentine. It is very likely that because of the distance many European scientists were absent from the congress.

For the same reason apparently, the exhibition of electro-physiological apparatus was comparatively small and a number of large firms did not participate at all.

No investigations on the physiology of the sense organs are carried out at the laboratories in Buenos Aires and Mendoza, with which we became familiar.

V. G. SAMSONOVA
*Translated by* M. J. KOPP

VOL
5
196

# ELECTRON SPIN RESONANCE SPECTRA OBSERVED ON ILLUMINATION OF PLANT LEAVES AND IN THE PHOTO-REDUCTION OF CHLOROPHYLL AND ITS ANALOGUES*

N. N. Bubnov, A. A. Krasnovskii, A. V. Umrikhina, V. F. Tsepalov and V. Ia. Shliapintokh

Institute of Chemical Physics, U.S.S.R. Academy of Sciences and the Bakh Institute of Biochemistry U.S.S.R. Academy of Sciences, Moscow

VOL. 5 1960

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The process of electron (hydrogen) transfer in photosynthesis is obviously associated with free radical formation both of chlorophyll itself and of its intermediate compounds since most redox reactions develop by one-electron transfers.

Wide use has recently been made of the method of electron spin resonance (e.s.r.) to detect free radicals. In a number of studies the e.s.r. method has been used to investigate the nature of the primary products of photosynthesis. Commoner [1] detected an e.s.r. signal in a suspension of chloroplasts and algae which was augmented five–six times on illumination although the spectrum of the signal in the light scarcely differed from that in the dark. The lifetime of the radicals induced by light was a few minutes. When dialysed chloroplasts were illuminated [2] the e.s.r. signal differed from that in the dark and from the signal observed on illumination of non-dialysed chloroplasts in the g-factor, the structure and width of lines and the lifetime. In the dark this signal disappeared within 5–10 sec. Calvin [3] in a number of papers has presented data on the detection of an e.s.r. signal in chloroplasts, the chromatophores of algae and the whole cells of various algae and bacteria when, illuminated —140° and —170°, i.e. in conditions in which the enzymatic reactions were completely inhibited. At low temperatures the signal persisted in the dark for several hours; with a rise in temperature to +25° the signal disapeared in the dark within 1–2 min, although the growth time of the signal at room and low temperatures was identical.

Apparently the free radicals found by Commoner and Calvin on illumination of the test objects are related to the formation of unstable products of photosynthesis. The nature of these radicals is not clear. It is most likely that on illumination several types of free radicals are formed differing in activity and lifetime. The authors of the papers suggest such a possibility.

The study of the radicals formed in the photochemical reactions of chlorophyll in model systems may help to establish the nature of the radical intermediates of the

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photochemical stage of photosynthesis. A model system in which the energy of light is stored in the photoproducts is provided by the reversible photochemical reduction of chlorophyll [4]. As spectral and electrometric measurements have shown [5, 6], photoreduction proceeds in the following elementary stages: $X + AH \rightarrow \dot{X}^- + \dot{A}H^+ \rightarrow \dot{X}H + \dot{A}$ where X represents the chlorophyll and AH the ascorbic acid.

The products formed by photoreduction can initiate the polymerization of methyl methacrylate [7]. Linschitz and Weissman [8] have shown that the "red" photoreduced form resulting from illumination of the chlorophyll-phenylhydrazine system in toluene does not give an e.s.r. signal. However, on direct illumination of the chlorophyll-ascorbic acid system in pyridine *in vacuo* in the resonator of the apparatus it was possible to observe the appearance of a low e.s.r. signal which disappeared on turning off the light [7]. Commoner [9] reported that he had been able to obtain an e.s.r. signal in the chlorophyll photoreduction reaction with ascorbic acid and that the signal corresponded to monodehydroascorbic acid.

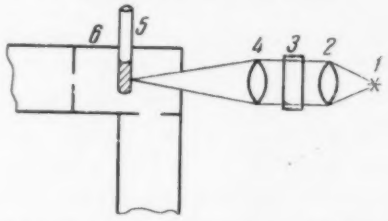


FIG. 1. Diagram of apparatus *1*, light source; *2* and *4*, lens; *3*, light filters; *5*, test object; *6*, resonator of e.s.r. spectrometer.

The present work was undertaken for the purpose of making a comparative study by the e.s.r. method of radical intermediates formed in the photochemical stage of photosynthesis and in model systems.

The experiments were carried out with an e.s.r. spectrometer with high frequency modulations of the magnetic field [10]. A resonator with an aperture through which light fell on to the sample was used to record the e.s.r. spectrum during illumination (Fig. 1). In the experiments the first derivative of the absorption line of the e.s.r. was recorded. As a light source we employed a 700 W projector lamp or a SVDSh-250 mercury quartz lamp plus a condenser. An infra-red filter was fixed in the path of the beam of light, i.e. a 10% $CaCl_2$ solution (thickness of layer 4 cm), and a filter to absorb ultra-violet, i.e. a saturated solution of sodium nitrite (thickness of layer 3 cm). In a number of experiments KS-11 and SZS-9 light filters were used.

## E.S.R. SPECTRUM ON ILLUMINATION OF PLANT LEAVES

The test objects were the leaves of the cereals *Triticum vulgare*, *Hordeum vulgare*, *Avena sativa* and the variegated leaves of the decorative plant *Sancheria*. The plants were grown in a hothouse.

Illumination of the leaves of *Sancheria* gave a signal with a wide band of complex structure. The parts of the leaf (containing and not containing chlorophyll) gave

different spectra. The long lifetime of the radicals formed (of the order of minutes) would indicate their low activity.

FIG. 2. E.s.r. spectrum produced by illumination of cereal leaves *1*, *Triticum vulgare*; *2*, *Hordeum vulgare* (at room temperature).

A quite different type of e.s.r. signal was observed on illuminating the leaves of *Triticum vulgare* and *Hordeum vulgare* (no signal was detected for *Avena sativa*) (Fig. 2). This signal which had been previously observed [11], is a doublet with a splitting $\Delta H \sim 1{\cdot}8$ oersted. The width of each component of the doublet is $\sim 1{\cdot}9$ oersted, the g value 2·004. This signal belongs to a much more active radical which disappears immediately the light is turned off. No e.s.r. signal was detected on illuminating the leaves through a KS-11 red filter.

Initially we assumed that the doublet signal was the result of the formation of a radical form of chlorophyll. To verify this assumption experiments were carried out in model systems in which we sought to obtain an e.s.r. signal of the reduced or oxidized radical forms of chlorophyll and allied pigments — the pheophytins *a* and *b*, haematoporphyrin and magnesium phthalocyanin. The experiments on the photooxidation of the pigments by molecular oxygen in pyridine or alcoholic solutions gave negative results. No e.s.r. signal was obtained for any of these pigments either at room or low temperatures (—30, —60°).

## E.S.R. SPECTRUM IN THE PHOTOREDUCTION OF CHLOROPHYLL AND RELATED PIGMENTS

Our experiments on the photoreduction of chlorophyll and its analogues were carried out in ampoules in the absence or in the presence of air. The dissolved oxygen was removed by evacuation and the ampoules sealed *in vacuo*. The ampoules were then placed in the resonator of the e.s.r. spectrometer where the photochemical reactions occured. In these experiments the ampoules were illuminated either with unfiltered light from a SVDSh-250 lamp or a 700 W projector lamp and a KS-11 red light filter was placed in the path of the beam of light in the experiments with chlorophyll, pheophytin and phthalocyanin. In the experiments with haematoporphyrin a CZS-9 blue-green light filter was incorporated. The signal was observed on illumination both with and without the light filters.

In experiments on the photoreduction of the pigments by ascorbic acid in pyridine solutions, an e.s.r. signal was obtained and the spectra of the signal depicted in Fig. 3 were coincident for all the pigments studied. Comparison of Fig. 2 and 3 also showed that the e.s.r. spectra of the living leaf and of the model system coincided both in the width of the doublet bands and in the splitting value. The values for the g-factor were

also concordant. The doublet signals were obtained in experiments on the photoreduction of the pigments in pyridine solutions. The controls showed that illumination of a pyridine solution of the pigment not containing ascorbic acid or of a solution of ascorbic acid in pyridine no e.s.r. signal was produced.

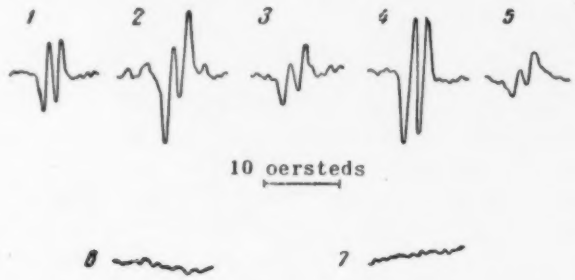


FIG. 3. E.s.r. spectra developing during photoreduction of pigments by ascorbic acid. Solvent — pyridine, concentration of pigment $10^{-3}$ M, concentration of ascorbic acid 1 M: room temperature; in experiments *1*, *2*, *5*, *6* and *7*, light filter — KS-11; in experiment *4*, light filter — SZS-9 was used. *1*, chlorophyll-*a*; *2*, chlorophyll-*a*+*b*; *3*, pheophytin *a*+*b*; *4*, haematoporphyrin; *5*, magnesium phthalocyanin; *6*, solution of ascorbic acid in pyridine without pigment; *7*, solution of chlorophyll in pyridine without ascorbic acid.

To find the components of the system associated with the appearance of the e.s.r. signal, we tested the photoreduction of the pigments in other solvents (toluene, ethanol) and with various reductants, namely, hydrazine, phenylhydrazine, thiourea, dihydroxymaleic acid, hydroxylamine and ferrous salts. Experiments were carried out with admixture of sodium hydroxide or acetic acid in order to increase the stability of the ion-radical forms. However, it was not possible in any of these experiments to obtain an e.s.r. signal. The parallel control tests to detect an e.s.r. signal from the photoreduction of eosin gave for any one of the reductants listed above in ethanol and pyridine a triplet signal characteristic of the reduced form of eosin [12]. Addition of alkali stabilized the semiquinone of eosin extending its lifetime from a few seconds to many hours.

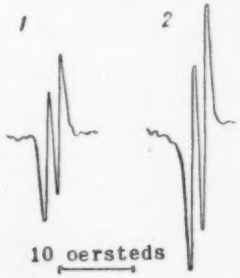


FIG. 4. E.s.r. spectra in response to illumination of piperidine solutions *1*, ascorbic acid; *2*, chlorophyll-*a*+*b* and ascorbic acid (Concentration of pigment $10^{-3}$ M, concentration of ascorbic acid 1 M; room temperature; red light filter KS-11 used in experiment with chlorophyll.)

We were able to establish the nature of the radical formed in the reaction between the pigment and ascorbic acid in experiments in which the photoreduction was carried out in piperidine solution; in this solvent the doublet signal was greatly amplified and this amplification of the e.s.r. signal in piperidine was noted for all the pigments. It should be pointed out that in the photoreduction of eosin in piperidine an intense doublet signal was obtained rather than the triplet signal of the semiquinone of eosin obtained in other solvents in the presence of ascorbic acid. A most important fact is that an intense doublet signal was obtained on illuminating a solution of ascorbic acid in piperidine not containing pigment (Fig. 4).

It should also be pointed out that after introducing ascorbic acid into the piperidine the solution assumed a greenish colour. The absorption maximum of the solution was 385 mμ which is apparently to be explained by a complexing of the ascorbic acid and piperidine. The appearance of the e.s.r. signal was in this case due to a direct photochemical process, probably by reason of the transfer of an electron in the coloured piperidine-ascorbic acid complex. In contrast, in solutions of ascorbic acid in pyridine, no radical of ascorbic acid was formed in the direct photoreaction — the radical was formed only as a result of the interaction of the excited pigment molecule and the molecule of ascorbic acid.

VOL. 5 1960

It must be supposed that the radical giving the doublet e.s.r. spectrum is a semi-oxidized form of ascorbic acid — monodehydroascorbic acid, since in the tested system reduction of the pigment and consequently the formation of the oxidized form of ascorbic acid occurs [4]. This also follows from experiments which showed that the signal appears in the presence of oxygen in systems containing ascorbic acid in pyridine in the presence of pigment and in piperidine whether in the presence or absence of pigment i.e. in conditions in which photo-oxidation of ascorbic acid is possible. The presence of oxygen in the solution containing the radicals does not decrease the intensity of the signal. At the same time in the presence of oxygen the reduced forms of the pigments do not occur and only sensitized photo-oxidation of ascorbic acid is observed [13]. The reaction of oxygen with the photoreduced forms of the pigment leads to a rapid regeneration of the pigment.

Thus, the experimental results indicate that on photoreduction of pigments by ascorbic acid, the signal observed belongs to the semi-oxidized radical form of ascorbic acid. The shape and width of the lines suggest that on illumination of the living leaf, pyridine solutions of the pigments or piperidine solutions either containing or not containing pigment we are recording one and the same compound — the oxidized radical form of ascorbic acid.

The reaction between the valency-saturated molecules proceeds according to the rules $0 \rightarrow 0$ or $0 \rightarrow 2$. This means that as a result of the reaction two free valencies are formed or, free valencies do not appear at all. Therefore, the appearance of the radical form of ascorbic acid in the reaction between the excited molecule of pigment and ascorbic acid in pyridine is indisputable evidence of the existence of the radical form of the pigments. The conclusion that radical forms of the pigments are produced in this system is also confirmed by the fact that in the presence of pigment the lifetime

of the radical of ascorbic acid is considerably shortened (in piperidine) from several minutes to several seconds, whereas in solution containing only ascorbic acid in piperidine the signal pe sists for a long time. This fact points to the possibility of a recombination of pigment and ascorbic acid radicals and also to the possibility of interaction between the ascorbic acid radical and the unchanged pigment. Thus, on introducing the chlorophyll solution into piperidine solution containing ascorbic acid radicals, the e.s.r. signal rapidly vanished (within 2–3 sec). Since in this reaction the monoradical interacts wi h a saturated molecule, the free valency cannot disappear. But in no experiment did we manage to detect an e.s.r. spectrum for the radical forms of the pigments, apparently because of their extremely high activity and corresponding short lifetime.

In the present work we were extremely interested in the question as to whether a radical of ascorbic acid is formed in the living leaf via the light absorbed by the chlorophyll or whether it arises through a direct photoreaction involving the ascorbic acid. An attempt to detect a spectrum produced by the formation of the radicals in the leaf was unsuccessful since the intensity of the e.s.r. absorption band even on irradiation with the integral unfiltered light of a mercury-quartz lamp was low. When the leaf was illuminated through a KS-11 light filter, allowing passage of the spectral region absorbed only by chlorophyll, no signal could be recorded at all, which might be due either to an appreciable decline in the intensity of the exciting light or to the fact that the radical is formed only when the leaves are illuminated in the short-wave portion of the spectrum. The experiments with etiolated leaves of wheat did not give reproducible results. Hence the question of the mechanism of formation of radicals of ascorbic acid in illuminated plant leaves is still unresolved.

VOL
5
196

## SUMMARY

(1) The method of electron spin resonance (e.s.r.) enabled us to detect the formation of free radicals during the photoreduction of chlorophyll-*a*, chlorophyll-*a*+*b*, pheophytin-*a*+*b*, haematoporphyrin and magnesium phthalocyanin by ascorbic acid and also on illumination of the leaves of several cereals. The structure of the e.s.r. spectrum was similar in all cases.

(2) The e.s.r. spectrum of the radicals detected both in model systems and in the leaves took the form of a doublet with splitting $\Delta H \sim 1{\cdot}8$ oersted, the value of the g-factor being 2·004.

(3) The e.s.r. spectrum obtained was that of the oxidized radical form of ascorbic acid (probably monodehydroascorbic acid) as suggested by the following facts: the structure of the spectrum did not vary with the nature of the pigment, the e.s.r. signal was observed only when using ascorbic acid as the hydrogen donor and appeared also in the direct photoreaction of ascorbic acid in piperidine.

(4) It has been established in model systems that the radical form of ascorbic acid may be produced both by reason of the energy of light absorbed by the pigment and by the direct photoreaction of the ascorbic acid bound to a strong base.

*Translated by* A. CROZY

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* There is a translation into English of this work.


VOL. 5 1960

# LONG-LIVED PHOSPHORESCENCE OF AROMATIC AMINO-ACIDS AND PROTEINS AT LOW TEMPERATURES *


IU. A. VLADIMIROV and F. F. LITVIN

Faculty of Fundamental Biology, Lomonosov State University, Moscow


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HOWEVER limited the number of papers devoted to the fluorescence of proteins it still exceeds the number of investigations of protein phosphorescence. Debye and Edwards [1] pointed out that the phosphorescence of proteins is determined by the aromatic amino-acids. Measurements of the spectra and duration of the phosphorescence of the aromatic amino-acids and certain proteins have been made by these workers and also by Steele and Szent-Györgyi [2] who established the position of the principal maximum of protein phosphorescence in the region of 440–450 mμ, this was assigned to tryptophan. The maximum excitation of protein phosphorescence was at 280 mμ [2]. The more detailed investigation of the spectra of long-lived phosphorescence (afterglow) and, in particular, the spectra for afterglow excitation is

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rendered difficult by the need for spectral measurements of the relatively weak glow excited in the far ultra-violet region — for which very sensitive light receptors are necessary. Luminescence methods, however, are highly rewarding in the study of the energy interactions between molecules and between different groups of the same macromolecule. While the absorption spectra tell us which groups absorb light, we have to judge which groups retain and luminesce the photon received — either themselves or by passing it on to an acceptor — from the luminescence excitation spectra; finally, study of the spectra of luminescence reveals which groups emit the photon absorbed by the macromolecule. A combination of these methods makes it possible to trace the fate of the photon in a multi-component system. Of the two types of luminescence, fluorescence and afterglow, study of the latter is more advantageous in that it allows detection of energy transfer (migration of energy) to the phosphorescent group not only between the singlet but also between the triplet states of the components of the given system [3].

We investigated the afterglow and the afterglow excitation spectra of the aromatic amino-acids: tyrosine, tryptophan and phenylalanine and of the proteins, zein, human serum albumin and egg albumin. In a number of cases the luminesence of the proteins was compared with that of equivalent mixtures of amino-acids in which the aromatic amino-acids were in the same relative concentrations as in the solution of the corresponding protein.

## METHODS

The measurements of the aftergow spectra were made on the apparatus previously used for measuring very weak luminosities [4]. The sensitivity of the apparatus when used as a photon counter was such that measurements could be made with relatively narrow slits: 0·02–0·04 mm. In investigating the spectra of excitation, the source of the exciting light was a hydrogen lamp: the monochromatic light at the output of the SF-4 quartz monochromator was focused by an aluminium mirror on the specimen. Scattered exciting light was absorbed by a ZhS-11 light filter when measuring afterglow, or by a UFS-2 + FS-6 filter in measurements of fluorescence. The luminescence was focused on the photocathode of the FEU-17 photomultiplier cooled in liquid nitrogen. Each measurement of afterglow involved three operations repeated two–four times: illuminating the object for 5 sec, closing the shutter for 1 sec, counting pulses from the photomultiplier for 5 sec. In order to increase the accuracy of the timing between the cessation of illumination of the sample and the switch on of the counting device an electronic time delay was used. The sample (solution of protein or amino-acid) was placed in a cooled metallic vessel with a quartz window. The temperature of the vessel was lowered to —150 – —170° by means of a heating element immersed in liquid nitrogen. The temperature of the object was measured with a thermocouple. The relative intensity of the exciting light was measured by a ZhS-9 fluorescent glass. In the experiments we used commercial preparations of amino-acids. The protein preparations were kindly placed at our disposal by A. A. Krasnovskii and A. G. Pasynskii. The amino-acids, the mixtures of them reproducing the proteins, and the albumins were dissolved in 0·1 M phosphate buffer and the zein in 60% ethanol.

## RESULTS AND DISCUSSION

Measurements of the spectra of low temperature luminescence of proteins and amino-acids may be liable to serious errors unless certain precautions are taken. The freezing of the solution in itself not only complicates the evaluation of the level of absorption and the relative quantum yields of luminescence of the substances, but in the case of the amino-acids creates the additional danger that crystals may separate out. Crystals of amino-acids apparently do not phosphoresce even at low temperature, but by absorbing the exciting light may distort the spectra of excitation of afterglow. At low concentrations of tryptophan (0·0004–0·002 mg/ml) practically all the luminescence transmitted by the ZhS-11 filter is afterglow. If we adopt as a criterion of the absence of "contamination" of the solution by crystals, the contribution of the afterglow of the sample to the total intensity of its luminescence and take this fraction as 100 per cent for the most dilute solutions, it will be seen with an increase in the concentration of the tryptophan solution from 0·0004–0·0020 mg/ml to 1 mg/ml, this fraction falls from 100 to 8 per cent, and equals 5 per cent for the dried, powdered amino-acid. Thus, the concentrations of amino-acids when measuring excitation spectra must be as low as possible. On the other hand, in investigating the afterglow spectra to strong ultra-violet light changes occurred in the afterglow spectrum of the specimen which were particularly noticeable especially in the case of very dilute solutions. The most intense photochemical processes obviously occur at room temperature, but solutions of amino-acids and proteins do not display afterglow at room temperature. In contrast, in the afterglow spectra off the dry protein — arachin — excited at room temperature by strong ultra-violet light the changes in the afterglow spectra were patently obvious being accompanied initially by a sharp accentuation of the maxima at 435 mμ and 455–460 mμ, then by the appearance of several new maxima and finally by discoloration and haziness of the spectrum. On lowering the temperature of the solutions to —150 – —170° the photochemical processes are naturally slowed down, although they do not completely cease, there was a gradual attenuation of the afterglow and minor changes in the form of the spectra of tryptophan and proteins in the 450–470 mμ region. These changes with temperature can be determined by repeating the measurements, taking the first measurement towards the longer and the second towards the shorter wavelengths. They were not very important in solutions with sufficiently high optical density (more than unity). In solutions of tyrosine and zein they might have affected the structure of the spectra.

The maintenance of the frozen solutions at a constant temperature from one experiment to another is of great importance for the reproducibility and correct interpretation of the results. It is probable that owing to the smaller difference in the position of the singlet and triplet levels of tyrosine as compared with tryptophan, the temperature dependence of the afterglow of tyrosine is characterized by a much steeper curve than in the case of tryptophan and therefore an increase in temperature leads at once to a decrease in the tyrosine component of the afterglow in the given sample.

Finally, it must be stressed that in the present work measurements were made only of afterglow prolonged for several seconds. Hence, the short afterglow of phenyla-

lanine (0·1 sec, according to Debye and Edwards [1]) was barely recordable in the dilute solutions of amino-acids and proteins.

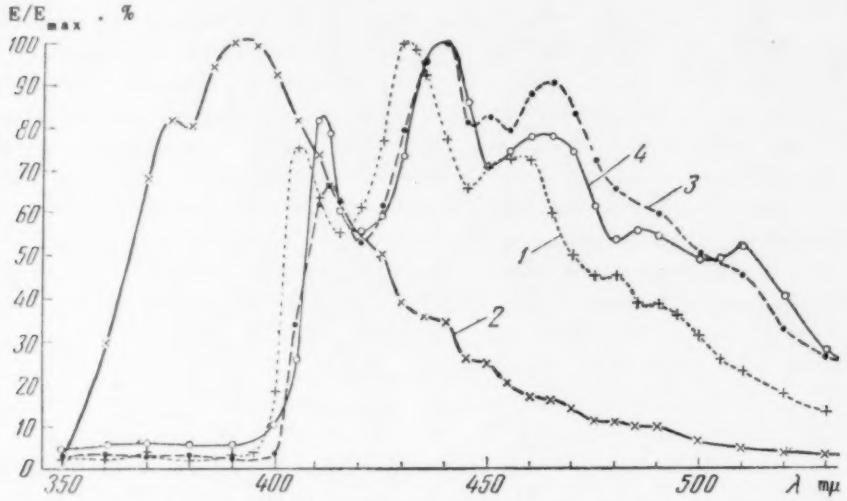


Fig. 1. Afterglow spectra. *1*, tryptophan; *2*, tyrosine; *3*, egg albumin; *4*, human serum albumin. E, intensity of afterglow in ergs/$\Delta\lambda$; pH 8·2.

The afterglow spectra of tryptophan, tyrosine, human serum albumin and egg albumin are depicted in Fig. 1. The figure shows:

(1) The afterglow spectra of the serum and egg albumins are apparently due solely to tryptophan.

(2) The main maxima of tryptophan in the protein (410–412 and 436–438 mμ) undergo a shift relative to the spectrum of the amino-acid in solution (460 and 430 mμ).

The shift in the main maxima of afterglow of tryptophan in the protein is probably to be attributed, as is the shift in the fluorescence maximum [5], to the difference in the state of the tryptophan in solution and in the polypeptide chain.

Much more surprising, at first sight, is the absence in the afterglow spectrum of albumin of the tyrosine maxima around 370–390 mμ. Egg albumin contains nine molecules of tyrosine and three of tryptophan per protein molecule; serum albumin contains the amino-acids in the ratio of 18 : 1 per protein molecule. Even allowing for a molar extinction ratio of 1 : 3 for tyrosine and tryptophan, the tyrosine contribution to the total absorption of the albumins is very high. On the other hand, the intensity of the afterglow of tyrosine in solution in our conditions was of the same order as for tryptophan, e.g. in one of the experiments with excitation of afterglow with a wavelength of 275 mμ, solutions of tyrosine and tryptophan with identical optical density of 0·2 showed in the same conditions the following intensity of afterglow: tyrosine (with a PS-9 light filter) 1900 counts/5 sec, tryptophan (ZhS-11 filter) 2570 counts/5 sec.

Thus, judging by the high proportion of absorption of tyrosine in the total absorption of exciting light by protein and by the large tyrosine afterglow in solution, the tyrosine component should have been significant in the afterglow spectra of the proteins studied by us. In fact, it dominated the spectrum of zein, but was absent from the spectra of egg and serum albumins; possibly for the following reasons: (1) decrease in the absorption of the protein-tyrosine as compared with tyrosine in solution; (2) reduction in the yield of luminescence of tyrosine when incorporated in a polypeptide chain of the protein; and (3) migration of energy from tyrosine to tryptophan.

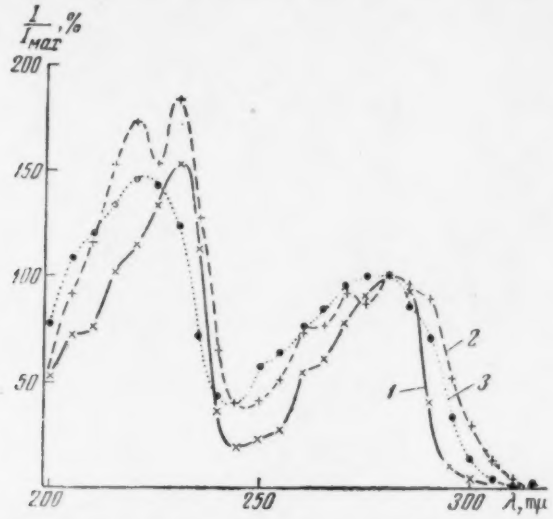


FIG. 2. Excitation spectra. *1*, afterglow of tyrosine; *2*, fluorescence of tryptophan at room temperature; *3*, afterglow of tryptophan; *I*, number of photons emitted by solution per photon of exciting light incident on the solution; pH 8·2.

Absorption of the aromatic amino-acids both in solution and as constituents of proteins is considered to be, if not exactly the same, almost virtually identical; this is, in fact, the basis for the method of spectrophotometric determination of these amino-acids in proteins [7]. Reduction in the luminescence yield of tyrosine when incorporated in the peptide chain of the proteins is very unlikely. Insulin [2] and zein [5] which do not contain tryptophan possess strong fluorescence, the spectra coinciding with the fluorescence of tyrosine; in our experiments at low temperatures zein showed a very strong afterglow, spectrally similar to that of tyrosine with a yield even somewhat higher than the afterglow yield of albumins. The most plausible explanation of these facts, as well as of the absence of the tyrosine and phenyalanine components in the fluorescent spectra of the proteins, is the migration of energy from tyrosine (and phenylalanine) to tryptophan [2, 5, 8, 9].

We shall now consider the results obtained from a study of the afterglow excitation spectra of the aromatic amino-acids and proteins (Figs. 2–5). These results may be summarized as follows:

(1) The excitation spectra of tyrosine and tryptophan were similar both in form and in the position of the maxima to their absorption spectra [10].

(2) The structure of the spectrum of excitation of afterglow and fluorescence of tryptophan was much more strongly pronounced than the absorption spectrum [6]. A possible reason for this is that the molecules of tryptophan which have the least interaction with the surrounding solvent molecules are those which luminesce and which, accordingly, show the most marked spectral structure (according to Thele and Weber [10] only 20 per cent of the tryptophan which absorbs light, in fact, fluoresces).

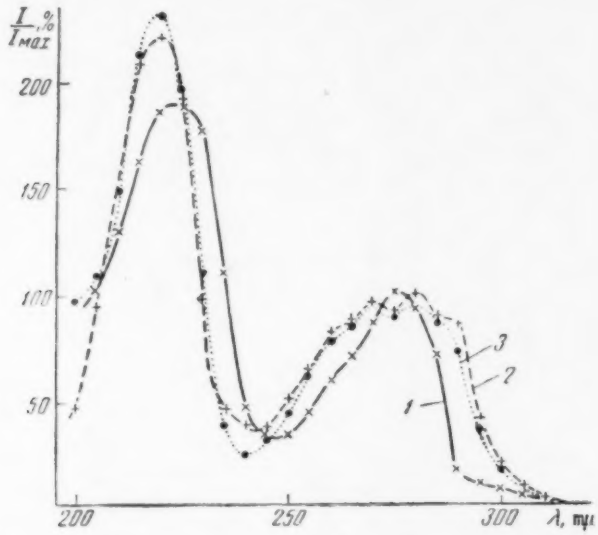


FIG. 3. Afterglow excitation spectra. *1*, zein; *2*, egg albumin; *3*, mixture of aromatic amino-acids in equivalent proportions to egg albumin; *I*, as in Fig. 2; pH 8·2.

(3) The afterglow excitation spectrum of zein (Fig. 3) was similar to that of tyrosine, but the maxima was shifted for the protein as compared with the amino-acid solution by 5–6 mμ to the longwave side, possibly because of the difference in the solvents (the protein was dissolved in 60% ethanol and the amino-acid in phosphate buffer).

(4) In the excitation spectrum of equivalent concentrations of the aromatic amino-acids of egg albumin the tyrosine component was present, but in the spectrum of the equivalent mixture for serum albumin it predominated (maximum 275 mμ) (Figs. 3 and 4).

(5) The afterglow excitation spectrum for egg albumin at pH 8·2 (Fig. 3), serum albumin at pH 8·2 (Fig. 4), and the fluorescence excitation spectrum for serum albumin at pH 5·9 (Figs. 5) was determined exclusively by the tryptophan (maxima 270, 280 and 290 mμ and minimum 275 mμ).

Hence, zein which does not contain tryptophan, showed a tyrosine component both in the luminescence and excitation spectra, whereas this component was completely

absent from the corresponding spectra not only of egg albumin but even of serum albumin which contains only one molecule of tryptophan per 18 molecules of tyrosine. This small tryptophan "impurity" quenches the luminescence of the tyrosine in the albumin molecules. However, this quenching is not accompanied by sensitization of the luminescence of tryptophan, since the tyrosine component is absent from the

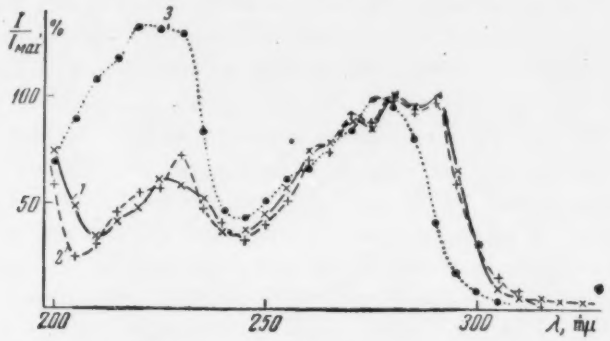


FIG. 4. Excitation spectra. *1*, afterglow of serum albumin; *2*, fluorescence of serum albumin at room temperature; *3*, afterglow of an equivalent mixture of aromatic amino-acids to serum albumin. *I*, as in Fig. 2; pH 8·2.

luminescence excitation spectrum of the protein. Possibly loss of the photon absorbed by the tyrosine occurs at the moment of transfer to an acceptor (tryptophan), in analagous fashion to that observed in solutions of dyes on concentration quenching of luminescence. Naturally, there is another simpler explanation, of the quenching of

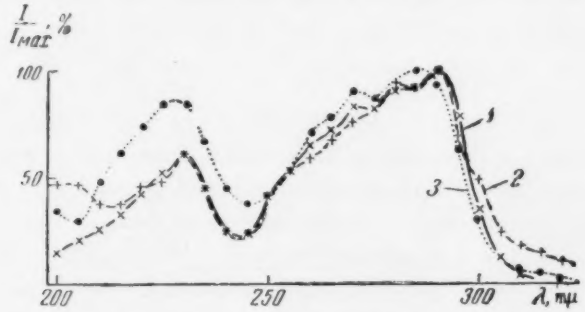


FIG. 5. Luminescence excitation spectra of serum albumin. *1*, afterglow; *2*, fluorescence at low temperature; *3*, fluorescence at room temperature; *I*, as in Fig. 2; pH 5·9.

luminescence of tyrosine in proteins, based on changes in its properties after incorporation into the peptide chain, but such an explanation is inconsistent with the intense fluorescence of the dipeptide glycyl-tyrosine and the strong fluorescence and afterglow of tyrosine in zein which does not contain tryptophan. Whatever the explanation, the photon absorbed by tyrosine is obviously converted into heat.

If the tyrosine present in protein converts the light energy absorbed into heat it must exert a screening action on protein fluorescence in concentrated solutions. As a result the relative quantum yield of luminescence of protein on excitation in the absorption region of tyrosine must be low as compared with solutions of tryptophan. On the basis of the data of the content of aromatic amino-acids [6] and the values of their molar extinctions [10] the value of the screening action of the tyrosine contained in the protein may be computed. Given complete loss of the photons absorbed by tyrosine, the luminescence yield of proteins on excitation with light of wavelength 275 mμ should be 55 per cent for egg albumin and for serum albumin 17 per cent of the yield of tryptophan in solution. In fact, in solutions completely absorbing the exciting light, the intensity of fluorescence of serum albumin on excitation by light of wavelength 275 mμ was only 37 per cent and for egg albumin only 61 per cent of the intensity of the fluorescence of tryptophan. The screening effect of tyrosine was thus for these proteins 76 and 86 per cent respectively of the theoretical values, but these discrepancies of 24 and 14 per cent might, to a certain degree, have been the result of differences in the fluorescence yields of tryptophan in solution and as a component of proteins.

(6) The shortwave maxima in the spectrum of excitation of the proteins were considerably less marked than in the spectra of the amino-acids, apparently because of the screening effect of the various non-fluorescent groups absorbing in this region (200–240 mμ). This is confirmed by the fact that in our experiments a rise in the concentration of protein reduced the shortwave component in the excitation spectra still further. Although the masking action of the strongly absorbing groups, e.g. tyrosine, may hinder the detection of minor effects, such a screening action, as opposed to sensitization, leads one to a rather sceptical approach to the possibility of energy migration to the tryptophan from groups which absorb in this region (peptide bonds, SH and S—S groups, etc.).

## SUMMARY

In an apparatus for investigating ultra-weak luminosities, mounted at the base of a photomultiplier cooled with liquid nitrogen and operated as a photon counter, the afterglow and the afterglow excitation spectra of proteins and aromatic amino-acids were investigated at a temperature between —160 – —170°. The afterglow excitation spectra of tyrosine and tryptophan were similar to the absorption spectra of these amino-acids but possessed a more definite structure. The excitation spectra of egg albumin and serum albumin, as with the afterglow spectra of these proteins, contained only the tryptophan component. In the corresponding spectra of mixtures of aromatic amino-acids reproducing the proteins, as with the spectra of zein which does not contain tryptophan, the tyrosine component was distinctly marked. It is assumed that in the proteins, migration of energy occurs from the tyrosine to the tryptophan accompanied by quenching of the luminescence at the moment of energy transfer.

VOL
5
196

In the afterglow excitation spectra of the proteins we did not detect any maxima which would indicate the migration of energy to the tryptophan from groups absorbing in the 200–250 mμ region (SH, S—S groups, peptide bonds, etc.).

Irradiation with intense ultra-violet light led to changes in the afterglow spectra of frozen solutions of proteins and amino-acids and in the spectrum of the dry protein — arachin.

In conclusion, we would like to express our deep gratitude to A. N. Terenin and A. A. Krasnovskii for their constant interest in the work.

*Translated by* A. Crozy

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VOL. 5 1960

# IONIC INTERRELATIONS AND THE ROLE OF POTASSIUM IN THE RHYTHMIC VARIATIONS IN THE INTRACELLULAR POTENTIAL OF INFUSORIA *

N. N. Kokina

Faculty of Fundamental Biology, Lomonosov State University, Moscow

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It can now be agreed that the contractile activity of the cilia of infusoria largely depends on the ionic composition of both the internal and external medium of the organism. Potassium exerts the most potent action on ciliary activity producing the so called reversal, i.e. a change in the direction of the ciliary beating [1]. An increase in the potassium content in the ambient medium causes a drop in the membrane potential of the cell [2, 3]. The nature of the dependence of the value of the membrane potential on the concentration of potassium shows that in the case of unicellular organisms it

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is due to the gradient of the potassium concentration around the membrane [4]. A direct relation has been found between the value of the membrane potential for the infusoria *Opalina ranarum* and the degree of reversal of its cilia [3]. It appears that an increased degree of reversal is accompanied by a drop in the membrane potential. The maintenance of a fixed level of the membrane potential corresponds to a constant direction of the ciliary beating, i.e. a constant direction of the metachronal wave. The degree of reversal of the cilia and the value of the fall in the membrane potential determine the state of excitation of the cell. The greater the reversal and the smaller the size of the membrane potential, the greater the degree of excitation of the cell at a given moment. Thus, potassium by causing ciliary reversal and a simultaneous drop in the membrane potential of the cell, leads to a state of excitation of the cell.

The action of potassium on the cilia and on the value of the membrane potential depends on a number of biochemical processes both within the cell and at its surface, e.g. the duration of the potassium-induced reversal may be greatly curtailed by the action of malonate which depresses the citric acid cycle [5].

The action of potassium is dependent on other ions. For example, preliminary adaptation of infusoria in a calcium-enriched solution, raises their sensitivity to potassium and extends the duration of the potassium-induced reversal [6]. Adaptation of the cell to a calcium-free solution considerably depresses the reversal and reduces the value of the drop in the membrane potential caused by potassium. These findings show that the increased concentration of intracellular calcium is a contributory factor in potassium-induced reversal. It is known that complete absence of calcium from the ambient medium inhibits the reversal due to potassium. This inhibition may be removed by adding small amounts of calcium to the solution [7]. The reversal is not observed on intracellular injection of potassium, although it may be induced by precipitation of intracellular calcium. All these facts led Komada [7] to postulate that reversal in the direction of ciliary beating in *Paramecium* depends on the ratio of the concentrations of potassium and calcium both in the internal and external medium of the organism.

The effect of potassium on the ciliary apparatus and on the membrane potential of infusoria, and the direct connexion between the magnitude of the potential and the degree of reversal suggest that potassium must play an essential role in the rhythmic variations of the membrane potential. This supposition also rests on evidence pointing to the constant participation of potassium in processes of excitation and automatism in multicellular animals.

The rhythmic variations in potential were first discovered by Kinosita [3] in the infusoria *Opalina ranarum*. He also found a relation between these variations and the rhythmical changes in the direction of ciliary beating.

A detailed study of these variations and their relationship to the various conditions of the external environment has been made by us in the Department of Animal Physiology of the Moscow State University [8, 9]. Our experiments also established a correlation between the direction of ciliary beating and the value of the membrane

potential. The membrane potential was measured at the moment of instantaneous fixation of the cell by the method of Parducz [10]. The cilia were frozen in the position they occupied at the moment of fixation and retained a definite pattern of the metachronal wave. The disposition of the cilia and the wave enabled us to determine the direction of their motion corresponding to the measured value of the potential. A number of such measurements showed that the value of the membrane potential drops with an increase in the degree of reversal.

This communication reports the results of a study of the influence of the ionic medium and in particular, of potassium ions on the rhythmic variations in the intercellular potential of the infusoria *Opalina ranarum*.

## METHODS

VOL. 5 1960

The test specimen — *Opalina ranarum* — is an infusoria which parasites the frog intestines. The intracellular potential was recorded by a microelectrode, using the method previovsly described [8]. The periodic variations in the membrane potential stabilized at a definite level 1–3 min after inserting the microelectrode in the cell. This stable background persisted for a long time (up to 2 hr) in the test solution for a constant rate of flow of the fluid through the chamber. If, in this period changes occurred in the shape of these variations, they developed so slowly that they did not impede the detection of the action of the various substances.

A test solution of the following composition was used as the medium for storing the cells before and during the experiment: KCl 125 mmole/l., NaCl 125 mmole/l., $CaCl_2$ $0{\cdot}7 \times 125$ mmole/l. mixed in a ratio of 2·5 : 100 : 3. In the experiments with potassium or calcium-free solutions the equivalent of these ions was replaced by sodium chloride. To raise the concentration of potassium or calcium in the medium, solutions of these substances in the above concentrations were mixed in the test solutions.

The osmotic pressure did not vary. The original potassium chloride solution (125 mmole/l.) was mixed with the test solution in a ratio of 1 : 0 to 1 : 200. i.e. the concentration of potassium was altered from 125 mmole/l. to 0·6 mmole/l., the potassium concentration of the original test solution (2·9 mmole/l.) was disregarded. The original calcium chloride solution ($0{\cdot}7 \times 125$ mmole/l.) was mixed with the test solution in the ratio of 1 : 0 to 1 : 40. The concentration of calcium in the test solution was 2·3 mmole/l. The solutions under investigation were introduced directly into the chamber containing the infusoria. The test solution was introduced into the same chamber to serve as control. The chamber capacity was 0·47 $cm^3$. The rate of flow of liquid through the chamber was constant in any one experiment but was varied from one experiment to another within the range 0·05 $cm^3$/min–0·25 $cm^3$/min. The substances under investigation did not enter the chamber containing the infusoria until several minutes after they had been placed in the flow. Before and after the action of these substance the cell was kept in the test salt solution. The direction of ciliary beating was observed under the microscope in the living cell and also in cells fixed by the method of Parducz.

## RESULTS

(1) *Influence of excess potassium on periodic activity.* In the first part of our work we studied the influence of excess potassium in the solution surrounding the cell on the rhythmic bioelectrical activity.

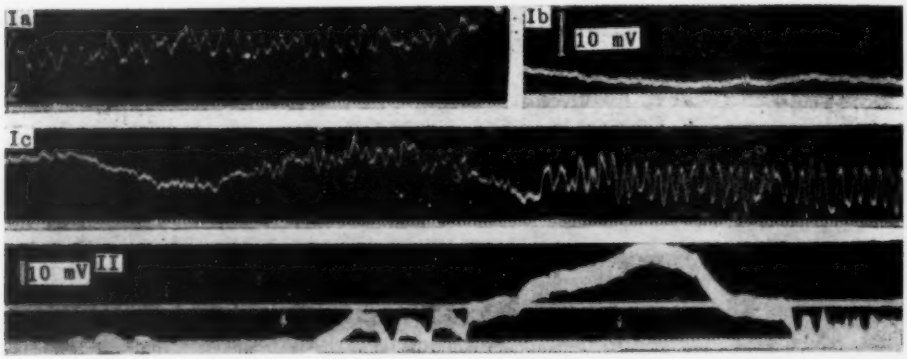


FIG. 1. *I*, Influence of potassium chloride (125 mmole/l.) on the value of the membrane potential and its periodic oscillations. *a*, Initial period of depolarization, with visible maintenance of periodic oscillations; *b*, subsequent complete depolarization—no variations in membrane potential. Time of action of potassium chloride 1 min. *c*, gradual restoration of periodic activity after washing away the potassium chloride solution, membrane potential already restored to normal. 1, record of membrane potential; 2, time trace (seconds). *II*, With rapid depolarization caused by potassium chloride the amplitude of the oscillations falls sharply but the rhythmic process is maintained. On washing away excess potassium chloride, the value of the potential and the character of the oscillations are restored. A step in potential is to be seen to the left on insertion of microelectrode into the cell. The arrows indicate the time at which substance introduced into chamber and the time at which it was washed away.

Potassium chloride, in a concentration varying from 125 mmole/l. to 31 mmole/l. produced marked depolarization of the cell membrane. The degree of depolarization varied with the potassium concentration. In a 125 mmole/l. solution of potassium chloride the membrane potential fell to zero. However, the insertion of the microelectrode into the cell in this solution caused a small rise in potential, of the order of several millivolts. Consequently, a small membrane potential is possibly maintained in this solution. The concentrations of potassium chloride which produced a drop in membrane potential completely inhibited its rhythmic variations. The inhibition of the periodic activity did not develop simultaneously with the drop in potential, but appeared later. When the potassium was washed off with test solution the value of the membrane potential returned to normal. The rhythmic variations reappeared only after a definite time lapse following the restoration of the potential (Fig. 1 (I)). Consequently, for inhibition of the periodic activity a definite period of action of the potassium, or a definite degree of depolarization is required. In some cases when the depolarization developed rapidly it was possible to see that the amplitude of the variations fell sharply, although the small pulses with the former rhythm persisted (Fig. 1(II)). Thus, the rhythmic nature of the process could persist for a certain length of time

even in conditions of depolarization of the membrane. Apparently, besides the depolarizing effect, potassium exerts an independent influence on the periodicity. Observations on the ciliar apparatus show that simultaneous with the drop in potential, reversal of ciliary beating set in. In the 125 mmole/l. solution of potassium chloride the harmony of the beating was disturbed over a large part of the cell surface.

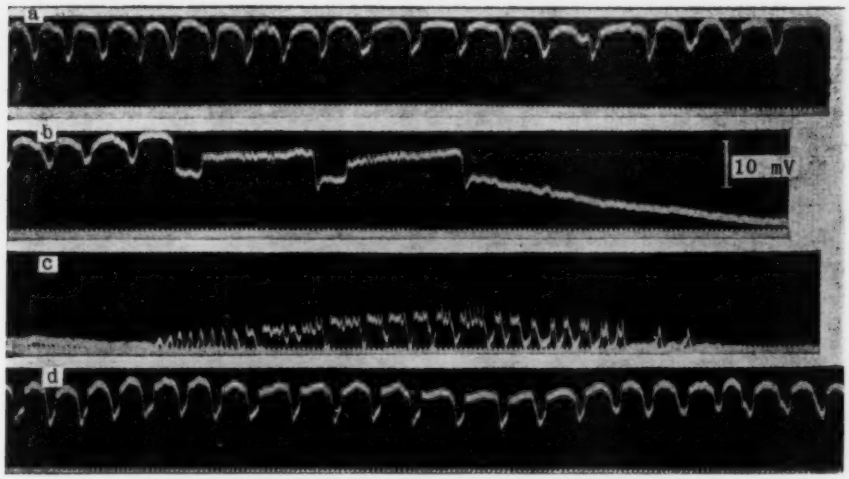


FIG. 2. Influence of potassium chloride (14 mmole/l.) on periodic variations in membrane potential *a*, character of oscillations in test solution; *b*, inhibition of periodic activity under influence of potassium; *c*, burst of periodic activity against background of inhibition; *d*, restoration of normal periodic pattern after washing away excess of potassium chloride. Symbols as in Fig. 1.

Solutions of potassium chloride in concentrations from 14 mmole/l. to 3 mmole/l. produced partial inhibition of the periodic activity (Fig. 2). The degree of inhibition fell with increase in the concentration of potassium. Together with inhibition of the slow waves, the effect of potassium was to make the rapid low voltage variations, which had a serrated form, more pronounced. We shall hereafter refer to these variations as the "potassium" form of the periodic activity. Similar variations were observed before recovery of the periodicity on washing off the higher concentrations of potassium (Fig. 1 (I)). On prolonged action of a surplus of potassium, the cell could pass from the state of inhibition even without washing off the substance. However, after a certain time inhibition again set in and then alternation of periods of inhibition and recovery was observed. Washing off the potassium with test solution gradually removed the potassium-induced inhibition (Fig. 2).

In some cases using 14 mmole/l. solution of potassium, inhibition was of a more persistent character. It lasted during the entire period of the action of potassium. The normal character of the rhythm was not restored on washing off. Against a background of this inhibition the "potassium" form of periodicity developed. Rapid, low voltage potentials appeared. However, in this case they were less regular. The

ciliary picture at this time was in the form of a number of dispersed groups of metachronal waves of different direction.

The small, rapid variations in potential which usually occured with potassium and sometimes in normal conditions are apparently a reflexion of local changes in the direction of ciliary beating in a small region of the cell. When one general wave pattern embraced the entire cell, no variations in potential occurred. In particular, this type of picture could be observed with uniform rotation of the cells when the potential remained constant. In this case, there were neither slow nor fast variations. The pattern of the metachronal wave at that time also remained constant. It may be assumed, from the data presented, that the slow potential variations are associated with a well-defined change in the direction of ciliary beating over the entire cell surface. The appearance of fast waves is obviously bound up with the occurrence of a local reversal of a small area of the cell surface, i.e. with less harmonious beating of the entire ciliary apparatus. Since potassium contributes to the appearance of the fast, small waves, it obviously stimulates the change in the metachronal waves and at the same time disturbs the harmonious rhythm of ciliary beating.

VOL
5
196

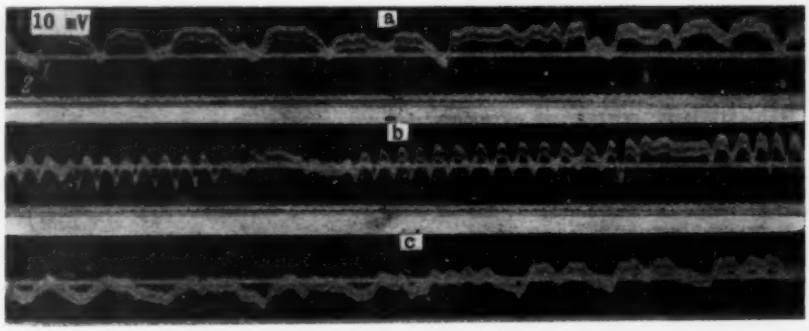


FIG. 3. Influence of potassium chloride (0·6 mmole/l.) on periodic variation in membrane potential. *a*, background activity in test solution; *b*, stimulation of periodic oscillations under influence of potassium; *c*, restoration of background activity after washing away excess of potassium. Arrow indicates time at which potassium chloride solution introduced into flow. Rate of flow of liquid, 0·24 cm³/min. Symbols as in Fig. 1. Time marking, 2 sec.

The inhibitory action of potassium on the periodic variations in potential decreased with concentration of the salt, the stimulatory action of potassium on the frequency of the periodic activity increased with decreasing concentration of potassium. It was to be expected that a slight surplus of potassium in solution would produce only a stimulatory effect. In fact, it was found that potassium at a concentration of 1·2 mmole/l.–0·6 mmole/l. considerably increased (doubled) the frequency of the periodic oscillations, without appreciable changing their amplitude (Fig. 3). Washing off the potassium restored the normal periodic activity. It should be noted that with a concentration of potassium of 0·6 mmole/l. the excess potassium in the medium surrounding the cell was equal to a fifth of its normal content in the test solution.

The influence on the periodic activity of such low concentrations of potassium suggests that the changes in the potassium level within the test solution must in itself affect the periodic variations in potential and may be involved in the regulation of these variations.

(2) *Influence on periodicity of changes in the potassium level within the limits of the test solution concentrations.* Prolonged washing (20 min and more) of the cell with potassium-free solution did not lead to any fundamental change in the rhythm pattern. It is possible that the potassium deficit of the cell in this case was supplemented by diffusion from the tip of the microelectrode. Therefore, to demonstrate the importance of small changes in the potassium concentration, we observed its action in relation to parallel changes in the calcium content of the ambient solution. It was found that the simultaneous removal from this solution of both ions (potassium and calcium) produced considerable depression of the rate of rhythm. Successive addition of both the ions helped to show the particular means by which they influenced the

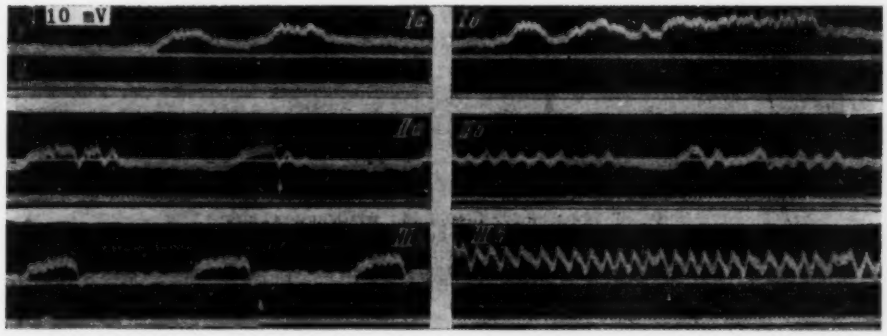


FIG. 4. Influence of potassium chloride of test solution on periodic variations in membrane potentials *Ia*, background activity in solution without potassium or calcium; *b*, change in activity 3·5 min after introduction of potassium into flow; *IIa*, background activity in potassium-free solution 7 min after introducing calcium into flow; *b*, change in periodic activity 1·5 min after introducing potassium into flow. Moment of introduction of potassium indicated by arrow. *III*, acceleration of periodics on washing off excess calcium (0·7 × 125 mmole/l.) by means of test solution; *a*, background activity in calcium solution; *b*, acceleration of periodics 3 min after introducing test solution into flow. Moment of introduction of te t solution indicated by arrow. Rate of flow of liquid, 0·07 $cm^3$/min. Symbols as in Fig. 1.

periodic activity. The action of potassium in these conditions varied according to whether it was introduced before or after introduction of the calcium. When introduced before the calcium it made practically no difference — all we could observe was a weakly marked increase in the number of low voltage spike potentials (Fig. 4 (I)). Only on addition of calcium was the disturbed periodicity restored. When the potassium was added to the sodium chloride solution after the calcium, its stimulatory role was more distinctly marked. The appearance of a large number of pulse peaks approximating in amplitude to the background slower oscillations was observed (Fig. 4 (II)). The increase in the number of pulses was observed also on addition of the potassium

to calcium-free solution but in this case they were very much less marked and their amplitude was very low. Thus, the presence of calcium caused an increase in the amplitude of the potassium effect.

This influence of calcium was more sharply marked in the following experimental conditions: the test solution flowing through the chamber was replaced for several minutes by an isotonic solution of $CaCl_2$ ($0{\cdot}7 \times 125$ mmole/l.). Washing away the excess calcium by means of test solution produced a constant, and distinctly marked sharp stimulation of the periodic activity (Fig. 4 (III)). This effect was not observed if the excess calcium was washed away with a solution not containing potassium, or if the potassium was added to potassium-free solution keeping the calcium level of the solution constant throughout the whole experiment. Stimulation was not always observed, and was much more weakly marked, if the addition of excess calcium and also its washing off were carried out at a constant level of potassium. The existence of this effect with a constant level of potassium and the absence of such an effect in calcium-free solution points to a relationship with both ions. An increase in the potassium level of the solution with a simultaneous decrease in the calcium content is obviously the best condition for promoting potassium stimulation.

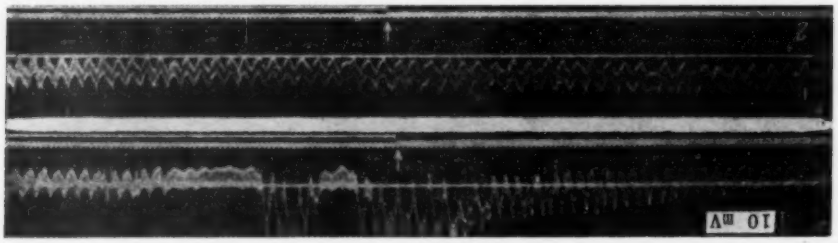

FIG. 5. Effect of excess calcium ($0{\cdot}7 \times 125$ mmole/l.) on periodic activity with prolonged washing off of potassium. *a*, increase in amplitude of oscillations 1 min after introduction of excess calcium into flow. Cell in potassium-free solution for 60 min. *b*, absence of effect 1 min after introduction of excess calcium into flow to the same cell. Time in potassium-free solution 35 min. Moment of introduction of test solution into flow indicated by arrow. Rate of flow of liquid 0·15 cm³/min. Symbols as in Fig. 1.

In all these instances of the stimulatory action of potassium it is clear that it exerted its influence primarily on the frequency of the rhythm-causing the appearance of characteristic spiked pulses of the "potassium" form. The amplitude of the potassium effect was determined by the changes in the calcium concentration in the solution. The calcium itself usually exerted no fundamental influence on the character of the rhythms. However, on replacement of the test solution by a calcium-free solution, the appearance of characteristic pulses with the same serrated form were observed, as in the case of an increased potassium concentration. These pulses vanished on restoration of the normal calcium concentration. Elimination of the pulsation caused by reducing the calcium level could be achieved by removing the potassium from the solution. This also confirms the stimulatory influence of potassium on the pulse frequency. The excess calcium can depress this form of pulsation if it exists in the normal

state. Sometimes with an increase in calcium we observed the appearance of slower waves or their acceleration and also a slight increase in their amplitude. Removal of calcium from the solution could lead to a decline in the amplitude and the frequency of the slow oscillations. The stimulatory effect of calcium on the amplitude could also be demonstrated by the action of the excess calcium on the cells kept for a long time in potassium-free solution (Fig. 5).

The results reported here show that the amplitude and frequency of the oscillations bear a direct relation to the ratio of the concentrations of both ions. A predominance of potassium in the solution produced an increase in the pulse frequency, while a preponderance of calcium helped to increase their amplitude. Given a state of equilibrium between both ions the optimum conditions are provided for manifestation of rhythmic electrical activity.

VOL. 5 1960

## SUMMARY

We have studied the influence of various concentrations of potassium in the ambient medium on the rhythmic periodic variations in the membrane potential of the infusoria parasite *Opelina ranarum*.

The results of the experiment show:

(1) Potassium chloride in a concentration of 125 mmole/l.–31 mmole/l. causes a drop in the membrane potential and depression of the rhythmic activity. These two processes do not have a parallel course. The periodic activity is inhibited after the start of depolarization and is restored, on washing away the potassium, only several minutes after the return of the potential to normal.

(2) Potassium chloride in a concentration of 14 mmole/l.–3 mmole/l. causes inhibition of the periodic oscillations. Against this background of inhibition, fast, low voltage spiked pulses appear, i.e. the "potassium" form of periodic activity.

(3) The inhibitory influence of potassium on the periodic activity decreases with reduction in the potassium level.

(4) Potassium chloride in a concentration of 1·2 mmole/l.–0·6 mmole/l. produces general acceleration of the periodic oscillations.

(5) The nature of the periodic variations in potential in the test solution depends on the ratio of the concentrations of potassium and calcium in this solution. Predominance of potassium causes the appearance of frequent, low voltage oscillations — the "potassium" form of the periodic pattern, whereas increase in the calcium concentration in the solution contributes to an increase in the amplitude of the oscillations. The addition of potassium to a potassium-free solution at the moment of washing away the excess calcium produces a general increase in the frequency of the oscillations.

(6) With a constant pattern of the metachronal wave neither fast nor slow variations in potential were observed. A changed value of the potential is connected with change in the direction of ciliary beating. Where potassium causes the intracellular potential to show haphazard and rapid low voltage oscillations, the ciliary picture takes the form of a series of scattered metachronal waves running in different directions.

It may be assumed from this that the "potassium" form of the periodic activity reflects the local changes in the direction of ciliary beating over a small area of cell surface.

The author wishes to express her deep gratitude to Prof. Kh. S. Koshtoiants for his constant guidance in this work.

*Translated by* A. CROZY

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VOL
5
196

# PERIELECTROTONUS AS THE BASIS AND MECHANISM OF PRODUCTION OF EXCITATION AND INHIBITION*

## (Biophysical Mechanism of Autovibratory Processes)

I. A. ARSHAVSKII

Institute of Normal and Pathological Physiology, U.S.S.R. Academy of Medical Sciences, Moscow

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THIS article gives in a condensed form a generalized account of the findings in our laboratory which make it possible from a unified monistic point of view, as first proposed by Vvdenskii (Wedensky), to understand both the mechanism of production of excitation and that of inhibition. The heart is a convenient test object for this purpose.

In 1930, Van der Pol [1] was apparently the first to notice that in general biological rhythms, physiological rhythms and, in particular, the rhythm of cardiac contractions represent typical examples of relaxation vibrations. He designed a physical model of an autovibratory system of the heart consisting of three systems of neon interrupters. As is known, Ukhtomskii [2] was greatly intrigued by the theory of nonlinear vibrations

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but at the same time he pointed out that Vvedenskii, in the laws of rhythmic excitation discovered by him, had established those typical features and laws peculiar to relaxation vibrations. Particular reference must be made to the work of Guliayev [3] who demonstrated the dependence of the rhythm of excitations produced in the nerve on

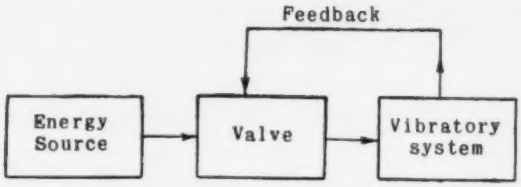


FIG. 1. Diagram of physical autovibratory system.

VOL. 5 1960

the laws of non-linear vibrations. The problem of specific physiological (and not physical) mechanisms determining the possibility of the automatic generation of rhythmic activity subject to non-linear laws is a most difficult one and still remains unsolved.

In the physical arrangement of an autovibratory system it is usual to distinguish: the source of the energy, the vibratory system proper and the valve, i.e. the organ regulating the entry of energy to the vibratory system (Fig. 1).

The valve and vibratory system are in a state of interaction, expressed by the fact that the valve controls the movement of the vibratory system and the movement of the vibratory system controls the work of the valve. As is known, such back action essential for any autovibratory system is known as feedback. By means of the feedback, in this case a positive feedback, automatic control is achieved as a result of which stable, sustained vibrations develop. In view of the fact that the physiological rhythms bear a striking resemblance to the autovibrations of corresponding physical systems, it would certainly not be without point to seek an analogy in living systems to the three elements mentioned as necessary for any autovibratory system. It is important to note that the generation of rhythmic activity in physical models may be achieved provided that these models constitute a combination of links forming a closed heterogenous system. Ukhtomskii [4] was the first to point to the importance of structural and functional heterogeneity (heterolability) as a condition for eliciting excitation in nerve elements. By inclusion of appropriate figures in his article, Ukhtomskii sought to illustrate the idea that the production of a rhythmic form of excitation is possible only at the boundary between two heterolabile formations. The rhythm of excitation is generated not in the actual nerve ending of the receptor, not in the nerve cell, not in the parabiotic focus, but at the boundary of the receptor ending with the dendrite, at the boundary of the nerve cell with the axon and at the boundary of the parabiotic focus and the adjacent unchanged nerve points.

Embryomorphologists and embryophysiologists long ago remarked that in mammalian embryos the first contractions of the heart tube occur not immediately on the appearance of the tissue of the myocardium, but with the appearance of the atypical tissue of the auriculo-ventricular node. As histologists have pointed out, the atypical

tissue of both the auriculo-ventricular and the sino-auricular node from the moment it first appears differs morphologically from the normal elements of the heart muscle.

In the present article on the basis of the activity of the heart and, in part, of the neuro-muscular apparatus we attempt to expound our conception of the specificity of the physiological mechanism both of generation of the rhythms of excitation and of inhibition.

We had the opportunity to observe the first excitations and the first contractions of the hearts of rabbit embryos at a stage between 9 and 10 days of their interuterine development. The contractions of the heart were recorded electrographically or electrocardiographically.

The following essential features can be observed in the auricle of rabbit, cat and dog embryos. By excizing part of the auricle wall even of a fragment with an area of 2–4 $mm^2$, we observed the continuing rhythmical movement of this segment, but only provided the so-called atypical tissue of the sino-auricular node was preserved within it. All the remaining parts of the auricles ceased to work. If, however, at the site of location of the atypical tissue a segment was cut out, smaller than the previous segment, so that this segment represented only the tissue of the sino-auricular node, no rhythmic contractions were observed — neither the muscle tissue of the auricles nor the atypical tissue of the sino-auricular node taken separately, could in itself perform any rhythmic contractions. The ability to display automatic periodicity developed and could only be observed when both tissue segments differing in their morphology, were connected by a certain interaction.

The facts described and observed in the embryonic heart can be reproduced with the frog heart. In the frog heart as in mammalian heart, two segments of atypical tissue are represented. One of them is located in the region of the sinus, the other is represented along the auriculo-ventricular border in the form of circular ring of muscle fibres. If the ventricle of the frog heart is separated by complete transverse sectioning, but in such a manner that in the ventricle there remains even a part of the atypical tissue of this ring, then as is well known, the auricle will continue to contract rhythmically. If the lower third of the ventricle is separated, without the atypical tissue, then the separated ventricle completely loses its ability to undergo rhythmic contractions.

Thus, the homogenous part of the muscle tissue of the apex of the ventricle is incapable of carrying out rhythmic excitations on its own; in the same way, as pointed out above, the homogenous part as such of the atypical tissue is incapable of carrying out rhythmic excitations.

Can we assume that the mechanism for performing rhythmic excitations is associated with the appearance and fixation during natural evolution of two heterogenous formations with a varying degree of lability — formations which interact with each other in one way or another? We first had to face this problem more than 20 years ago in connexion with our observations on the cathodic and anodic polarization of the frog heart. This problem again cropped up in our analysis of characteristic features of the activity of the heart in mammalian embryos. In natural conditions the rhythmic

excitations in the heart develop only from the time of appearance of the area of atypical tissue. If in experimental conditions we can produce artificially in the homogenous lower third of the ventricle of the frog heart a small region of impairment, then is it not possible that we shall obtain a mechanism reproducing the rhythms of the isolated apex of the ventricle?

In 1936, we published data showing how the phenomenon of perielectrotonus in the ventricle of the frog heart with local impairment in the ventricle by the action of a d.c. cathode or anode could be visualized [5]. At that time our attention was attracted by the fact that local impairment in the isolated apex of the ventricle by a d.c. cathode reproduced in it rhythmic contractions. In recent years, we have again turned our attention to these observations. The experimental arrangement was as follows. The isolated apex of the frog heart embedded in paraffin, was placed in a Petri dish and covered by a round sheet of filter paper moistened with saline. The tip of one of the non-polarisable electrodes — the indifferent one — maintained contact with one portion of the filter paper to the side of the isolated apex. The tip of the other electrode was in local contact with a part of the apex of the heart. Along the axis of the apex two needles were inserted at corresponding sites in the filter paper to pick up the action potentials which were recorded on an oscillograph. As the observations showed, the production of a local area of impairment, not only by the d.c. cathode but also by the anode, restored the ability of the apex of the heart to carry out the automatic rhythm of excitation. A definite intensity of the polarizing current is necessary to produce this capacity. At first a threshold of intensity was found at which the apex of the heart responded only with a single contraction to the make and break of the d.c. current. This intensity was not sufficient to produce repetitive automatic periodicity. With a further increase in intensity, the apex was able to respond not only with a single, but with a rhythmic series of excitations. The automatic reproduction of the excitations then lasted only for a certain time irrespective of the continuing action of the d.c. current. As a result of the sustained action of the current, the automatism was again blocked.

If for the experimental reproduction of automaticity it suffices to produce a local area of impairment anywhere in the homogenous tissue of the apex of the heart, it is obvious that this need not necessarily be achieved by a d.c. cathode or anode. By applying close to the base of the excized apex of the frog heart a crystalline particle of sodium chloride, we observed, as a rule, after a certain latent period, the appearance of an automatic series of excitations. The rhythm of the excitations persisted from 1 to 3–4 min. With the sustained action of the salt crystal the automatism was later blocked as in the case of the action of a direct current (Fig. 2).

Fig. 2 reproduces the curves of the biphasic action potentials with the contiguous succeeding potentials recorded for the isolated apex if a crystal of salt was applied to its base. In recording the action potentials the tip of one recording electrode was in direct contact with the crystal i.e. through the crystalline particle with the artifically-produced leading node; the tip of the other recording electrode was situated at the centre or the end of the isolated apex. The action potentials recorded were of the same

character as those previously recorded at the isolated apex of the ventricle of the heart with the atypical tissue maintained in the ventricle. Thus, in natural conditions morphological and hence functional continuity between the segment of atypical tissue and the rest of the myocardium is essential for the production of the automatic rhythm of excitations. In experimental conditions, for the production of the automatic rhythm of excitations functional heterogeneity between the local segment of impairement and the rest of the myocardium is necessary.

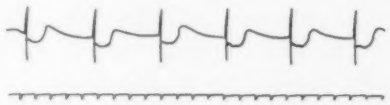

FIG. 2. Oscillogram of automatic rhythm of excitations in isolated apex of frog heart. Leading node of heart produced by the application of a crystal of sodium chloride to the apex.

These physiological studies have demonstrated that chemical stimulation or passage of a direct current through the isolated apex of the frog heart produces a rhythmical series of contractions. It suffices to recall the experiments of Trendelenburg [6]. The results of these experiments have been interpreted as supporting the point of view that in natural conditions the automatic rhythm of excitations is the product not of periodic, but of continuous stimulation. These experiments have not been used to analyse and understand the very essence and nature of automatism. By what mechanism does the heart function as an autovibratory system? To answer this question we shall refer to the following series of observations.

In determining the chronaxie of the frog heart we find that its value is more or less uniform varying from 2·8 to 3·2 msec irrespective of the site in the ventricle at which the determination is made. If the anterior surface of the ventricle wall is subjected to local cathode impairment with non-polarisable electrodes, then with a potential difference between the electrodes of about 1 V, the following phenomena can be observed. Directly under the electrode, and in strict conformity with its shape, an area of depolarization is formed producing relaxation of the impaired part of the myocardium and its exclusion from systolic activity. The result of the depolarization of this region is a lengthening in the chronaxie to 5–6 msec and more. Next to the electrode the state of polarization of the myocardium is enhanced. This is expressed in a certain pallor of the ventricle and a shortening of the chronaxie to 2·0–1·8 msec. We are thus faced with the phenomenon which Vvedenskii [7] described in 1901 as associated change of excitability in a periparabiotic region and which in 1920 he termed perielectrotonus.

With local anodic polarization of the same intensity we also observed the phenomenon of associated perielectrotonic contrast, but of a reverse nature. Directly under the anode the ventricle became pale and its polarization rose, reflected in a shortening of the chronaxie to 1·8–1·6 msec. Around the anode a rim of red swelling was formed — state of depolarization expressed in a lengthening of the chronaxie to 5–6 msec. With the given power, namely, within the limits of about 1 V between the electrodes con-

ditions arose for the automatic rhythm of excitation in the isolated apex. Merely from the picture of the variations described it will be understood why the automatic rhythm of excitations developed both with cathodic and anodic impairment. In producing a stationary biopotential with the d.c. cathode or anode we by no means obtained the unambiguous state commonly believed right up to recent times, to exist in electrophysiology. The state which developed embraced components of electronegativity and electropositivity simultaneously located in space and combined with each other in contrasting fashion, i.e. both catelectrotonic and anelectrotonic components simultaneously.

The observations made regarding galvanic impairment also hold good for cases of chemical impairment. All that we know from the Vvedenskii—Ukhtomskii school supports the belief that by applying a crystal of salt to the isolated ventricle, we should produce directly under the crystalline particle depolarization and an associated rise in polarization in adjacent areas. Thus, in the case of chemical impairment also we create a state involving catelectrotonic and anelectrotonic components. The associated perielectrotonic contrast formed and developed under the influence of impairment must of necessity reach a certain level at which the apex of the heart is able to achieve an automatic rhythm of excitation. If the requisite level of associated contrast is not reached, or if it is surpassed, then automatic periodicity is not brought about. When on cathode impairment in the ventricle the voltage between the electrodes equals 2 and 3 V, then in the area of depolarization produced directly under the cathode, the chronaxie is indeterminable, since the rheobase cannot be determined because of the complete loss of excitability in this area. On the other hand, in the adjoining area, the state of polarization is raised very considerably and is reflected in a shortening of the chronaxie to 1·5 and even 1·2 msec. Although in this case the associated contrast becomes very considerable the level of contrast reached is such that system is blocked or shut off. It is thus understandable why with persistent galvanic or chemical impairment the automatic rhythm of excitations developed ceases after a certain time.

The experiments discussed are analagous to our observations of the arrest of cardiac activity induced by stimulation of the vagus. In our experiments on the dog heart with the thoracic cavity exposed and with the use of artificial respiration, then on stimulation of the peripheral segment of the vagus, we noted the sharp and considerable increase in the chronaxie in the sino-auricular node. In some experiments the chronaxie showed such a considerable increase that it could not be determined. As against this, in the myocardium of the ventricle the chronaxie shortened. At the same time we observed the development of electronegativity in the sino-auricular node and a perielectrotonic associated state of electropositivity in the myocardium of the ventricle [8]. Thus, the mechanism of shutting off or inhibiting the activity of the heart by stimulating the vagus is the same as the mechanism of shutting off or blocking the automatic rhythm of excitations in the isolated apex of the heart. This mechanism is based on the production of that degree of depolarization in the pace-maker, and on a simultaneous rise in the polarization over the remainder of the myocardium, at which the automatic generation of rhythmic excitations spreading in the form of waves becomes impossible.

Recently a design of experiment has been found which makes it possible to observe electrographically the associated perielectrotonic contrast which forms the basis for the automatic rhythm of the cardiac excitations. The experiment is carried out as follows. A metal ring used as an indifferent electrode is placed on a sheet of filter paper

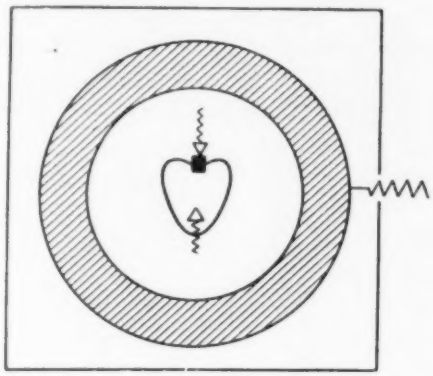

FIG. 3. Diagram of the apparatus for recording the variations in potentials during the cardiac cycle. Potential measured in the portion of tissue corresponding to the node artificially produced by the crystal of sodium chloride (black square) and separately in the tissue of myocardium.

(Fig. 3). The other electrodes are arranged as follows: one on the crystalline particle, i.e. on an artificially produced leading node, the second at a given site of the myocardium of the isolated apex. The differential recording of the potentials developing in one or other of the elements is made by means of a two-channel oscillograph. Fig. 4 shows the curves of the action potentials thus recorded.

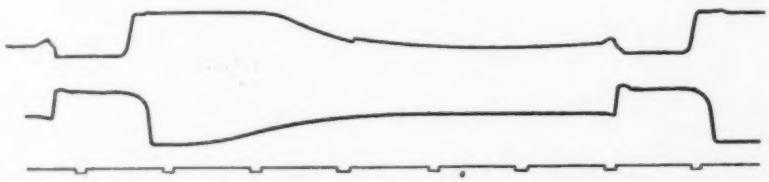

FIG. 4. (*a*) and (*b*). Upper electrogram shows variations in potential in the area of impairment (pick-up through crystal of sodium chloride). Lower electrogram variations in potential in myocardium at a distance of 4 mm from area of impairment.

The upper curve shows the action potentials of the artifically produced leading node, the lower curve the action potentials of the myocardium of the isolated apex. It will be seen that whereas in the node negativity develops, positivity develops at the same time in the myocardium, and the converse is also true. One curve is, as it were the mirror image of the other. In the present communication, we cannot go into the very important and essential details disclosed by electrographic recording of the asso-

ciated perielectrotonic contrast in two interacting elements. The curve depicted shows two phases or two levels of associated contrast. In the first, a less pronounced level of associated contrast of the system is observed and excitation is brought about. In the second, the more pronounced level of associated contrast of the system becomes refractory, it is shut off.

The specificity of the physiological mechanism responsible for the autovibratory character of cardiac activity is brought about by the associated interaction of the two elements. One leading node performs the function of a valve and is marked by a lower lability. The other is the myocardium performing the actual work of the vibratory system and is characterized by signs of higher lability. The typical feature of a physical autovibratory system is compensation of the losses by the energy source. In an electrical vibratory system; in a valve generator, in a neon interrupter, etc. the source of energy is the conventional galvanic battery. In the autovibratory system of the heart each of the tissue elements ("capacitors") mentioned combines within it, not only the functions of a valve or a vibratory system, but also the functions of an energy source. Because of this the automatic process of carrying out rhythmic non-linear excitations is realized, thanks to the periodic exchange of the store of energy or more accurately the periodic induction of the compensation energy between both elements. The discharge of energy in one tissue capacitor, namely in the valve, also involves an associated increase in the store of energy in the other tissue capacitor — the working vibratory system. Discharge of energy in the vibratory system causes an associated increase in the store of energy in the valve thereby compensating for previous losses.

It may be assumed from our findings that metabolism in the homogenous tissue substrate cannot in itself be the source of the generation of the autovibratory process. In this sense, our conception of the mechanisms of production of autovibrations differs from the conception propounded by Zubkov in his highly interesting paper [9]. From our point of view, the importance of metabolism lies in the periodic recovery of that structural heterogeneity between two dissimilar tissue formations in contact which is necessary as the source of generation of autovibrations. What is the mechanism of the specific feedback whereby the interaction between the two tissue elements comes about: in the case in question — between the atypical tissue of the leading node and the myocardium?

At present we can only answer in the most general terms by stating that such a mechanism is associated perielectrotonic induction. Thanks to this mechanism of mutually associated perielectrotonic induction between two tissue elements, selfregulatory control is achieved as a result of which stable, sustained rhythmic excitations in the heart and in any other excitable heterogenous system may appear. However, it must be noted that the stable, sustained generation of periodic activity and the exchange of energies can occur only in a vibratory circuit consisting of capacitance and self-induction. In a circuit consisting of a combination of two capacitors, the generation of a sustained rhythm of vibrations cannot take place. However, no tissue system generating rhythmic excitations can be conceived as consisting of a combination of two such elements as self-induction and capacitance.

It is important to remember that perielectrotonic interaction is carried out between two elements of which the intermediate link (valve) has a negligible mass as compared with that of the functioning vibratory system.

It would appear that the most realistic approach to associated perielectrotonic contrast between these two elements — the atypical tissue of the leading node and the myocardium — is to assume that these elements function in the manner of semiconductors with a junction between them. By assuming that the tissue elements in

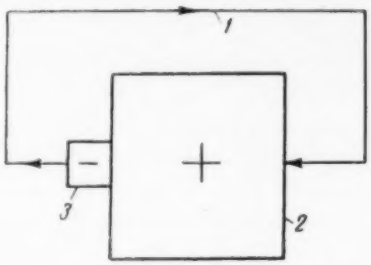


FIG. 5. Established distribution of charges and potentials in semiconductors in contact. *1*, feedback; *2*, myocardium (functioning vibratory system); *3*, (valve) leading node of heart.

contact are characterized by the properties of semiconductors, we can understand not only the mechanism of formation of the associated perielectrotonic contrast, but the mechanism of its formation at the boundary of the two elements, the mass of one being hundreds and even thousands of times smaller than that of the other (Fig. 5).

By adopting the concept of a semiconductor junction we are able to understand why in the mechanisms of production of inhibition established by us, negativity comes about in the intermediate link (valve), whereas in the actual functioning vibratory element positivity is realized, combined with storage in this element of potential energy.

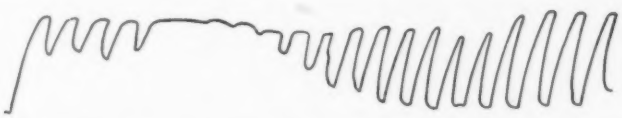

FIG. 6. An example of the production of highly regulated periodic contractions of the muscle in response to indirect stimulation with stimuli (from an electron stimulator) of definite duration, amplitude and frequency.

If the unique perielectrotonic mechanism established by us for carrying out both the process of excitation and that of inhibition is correct, it is obvious that in a neuromuscular system, it is possible in principle that for the intermediate link (neuro-muscular junction) such properties will be found in which sustained autovibratory periodic processes may be initiated (analogous to the periodic autovibratory activity of the heart). Such conditions have been found in our laboratory by M. N. Kondrashova and I. A. Korniyenko using indirect stimulation of the gastrocnemius muscle of the

rat with an electron stimulator giving rectangular stimuli of definite amplitude, duration and frequency. After selecting a definite form of indirect stimulation we were able to see how the muscle at the height of the induced tetanus passes into periodic relaxation and contraction. The muscle can respond to continuous stimulation of the nerve with a sustained autovibratory rhythm of tetani for an hour and more (Fig. 6).

The mechanism of execution of the autovibratory process discovered, i.e. excitation and inhibition at the periphery, provides a means of deepening our understanding of the mechanisms of execution of excitation and inhibition in the nervous system functioning as an autovibratory (non-linear) system [10–11].

*Translated by* A. CROZY

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# ROLE OF THE NERVOUS COMPONENT IN DARK ADAPTATION*

V. D. GLEZER, R. G. MANGUSHEV and A. B. ATLAVIN

Pavlov Institute of Physiology, Leningrad

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IN recent years a number of workers [1, 2] have drawn attention to the fact that the photochemical theory of adaptation is at variance with certain experimental data, namely, the changes in the concentration of visual purple in various systems of illumination do not correspond to changes in sensitivity to light. The hypothesis has been advanced that a major rôle is played by the nerve factor in the changes of light sensitivity on adaptation [3–9]. According to this hypothesis a rise in sensitivity on dark adaptation is associated with an increased ability for spatial summation. In turn the increase in spatial summation is the result of an increase in the receptive field.

* Biofizika **5**: No. 2, 152–157, 1960.

An increase in the receptive field in dark adaptation while increasing light sensitivity, leads at the same time to diminished visual acuity. Such a reciprocity of visual acuity for light sensitivity in various circumstances is of great adaptatory biological importance in the life of the the animal, imparting to the visual analyser, as the most important system in man connecting the brain to the external environment, the features of a communication system with optimal coding [10]. The present article concerns some experimental data confirming within certain limits the hypothesis of the rôle in adaptation of the summation processes in the retina, and the interdependence of spatial summation and visual acuity.

The following considerations formed the starting point of our work. Glezer [11], in investigating the dependence of the size of the receptive field on the region of the retina in conditions of dark adaptation, demonstrated that outside the fovea the size of the rod receptive field does not appreciably change and only slightly increases at the extreme periphery of the eye. Thus, at 2°50′ distant from the centre of the eye the diameter of the receptive field was 61′ while at 40° it was 77′.

It is also known [12] that at 3° distant from the centre of the eye visual acuity changes considerably with the luminance of the background, i.e. with changes in adaptation: with a change of the latter from 9·0 to 4·9 log μl visual acuity is reduced from 0·38 to 0·015, i.e. by a factor of 25. At the same time at 30° visual acuity changes but little and with the same alteration in luminance of the background is reduced only by a factor of 1·5: from 0·032 to 0·020. The sizes of the rod receptive fields in conditions of dark adaptation [11] coincide with those of the object to be resolved,* this is a further proof of the validity of our original hypothesis.

The following experimental approach to the problem was suggested by an analysis of the above-mentioned facts. If visual acuity is really associated with a change in the summation properties of the functional unit of the retina, i.e. with a change in the size of the receptive field, then changes in summation as a function of adaptation must be quite different in the parafoveal region and at the extreme periphery of the retina. On the basis of these considerations the curves of dark adaptation in two regions separated from the centre of the eye by 2°50′ and 40° were plotted. The curves were recorded for round test objects of different size: test object No. 1 had a diameter of 55′, No. 2 of 9·5′, No. 3 of 2·6′.

The size of test object No. 1 roughly coincided with the maximum for the rod receptive field; test object No. 2 was of a size corresponding to the maximum known value for the cone receptive field in the foveal region [11]. The investigations were conducted as follows. After 30 min dark adaptation the subject was exposed for 10 min to an intensity of 2500 asb, by means of a sphere, for preliminary light adaptation. After this the curves of dark adaptation were recorded on an ADM adaptometer (modified model).

* This interesting fact indirectly supports the assumption that the rod receptive fields unlike those of the cones, do not overlap.

Three investigations were carried out for each of the three test objects at angular distances of 2°50′ and 40° from the point of fixation, a small red point at the threshold of visibility was used for this purpose.

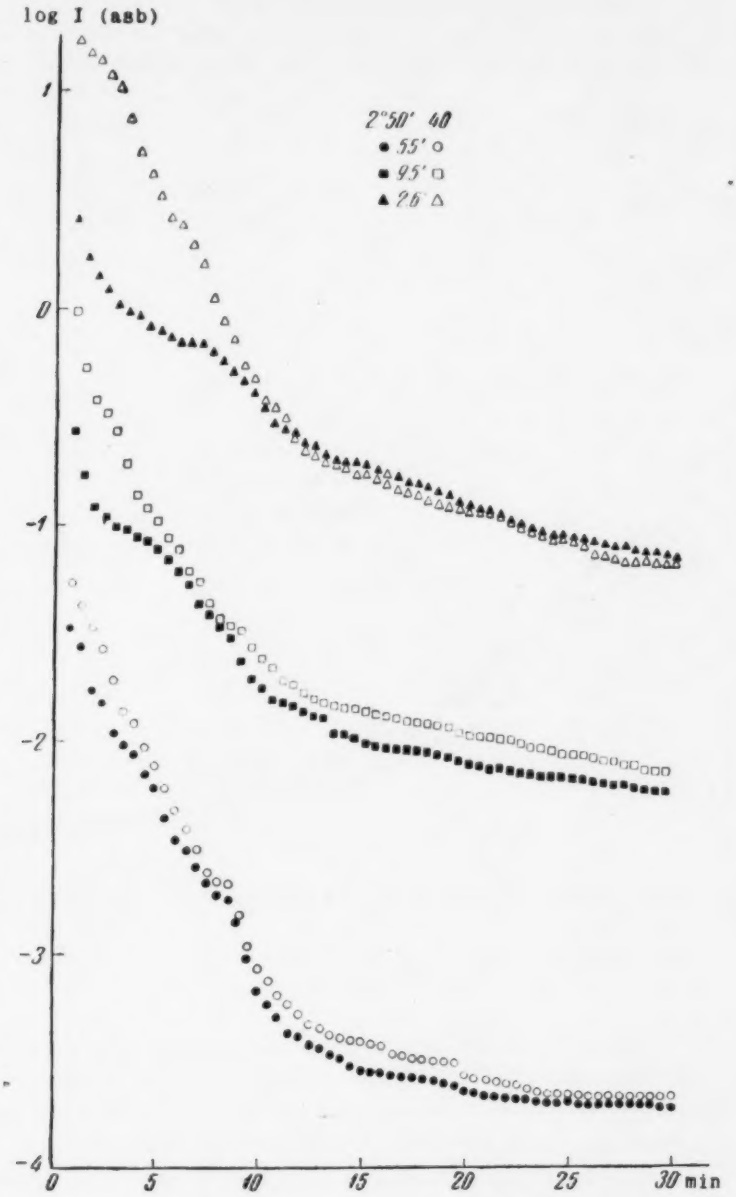


FIG. 1. Variation of the course of dark adaptation with the region of the retina and magnitude of the light stimulus.

Fig. 1 reproduces the averaged curves of dark adaptation obtained from one subject. Similar results were obtained for the second subject.

The following fact is noteworthy. In those pairs of curves (the black symbols correspond to an angular separation of the point of fixation and the test object of 2°50′, the open to 40°), where the stimuli were small, a divergence was observed in the first stage of dark adaptation (before the inflexion in the course of the curves). This stage,

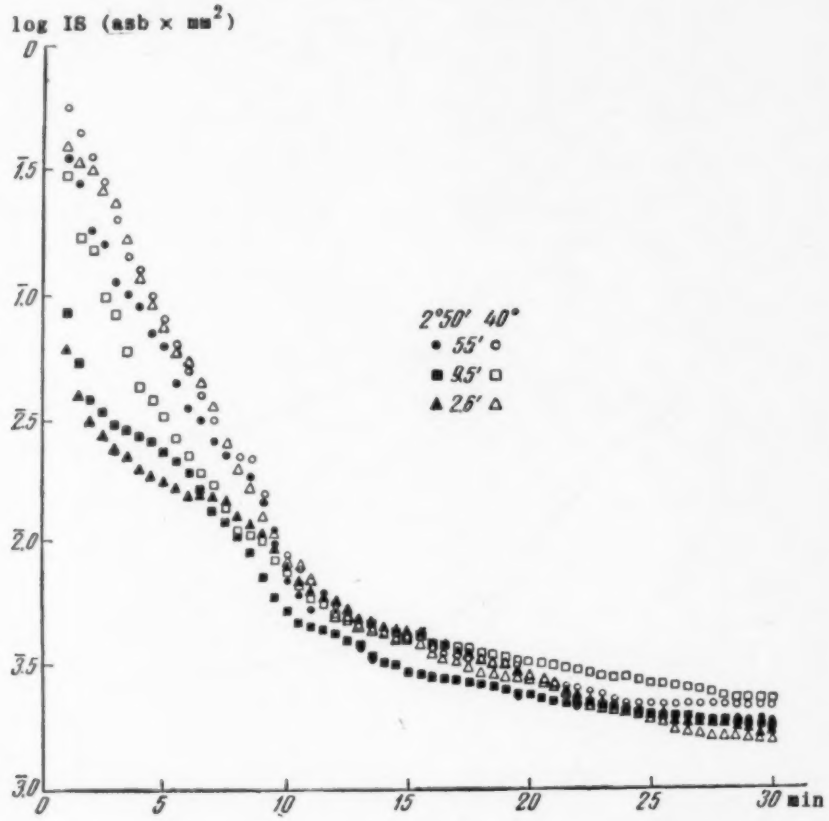


FIG. 2. Variation of threshold quantity of light with time of dark adaptation in different regions of the retina and for different values of light stimulus.

as is known, is associated with changes in the sensitivity of the cones. In the second stage of adaptation (changes in rod sensitivity) the curves run parallel. However, for the purposes of discussion, it is more convenient to represent these data as the variation of threshold energy with time of adaptation (Fig. 2).

Before analysing this relation, we shall consider how the magnitude of the stimulus affects the determination of light sensitivity. Provided the area of the light stimulus is equal or less than the area of the test receiptive field, changes in the size of the stim-

ulus do not affect the value of the threshold energy, since the Ricco law applies within the receptive field. If, however, the area of the stimulus is greater than that of the receptive field, then a considerable part of the energy impinging on the retina is wasted (provided that the light energy falling outside the receptive field is insufficient to elicit excitation in adjacent receptive fields).

We shall consider the curves in Fig. 2 from the point of view of vision. The right hand rod branches of the curves, rod vision, (starting from 7–9 min dark adaptation) virtually coincide irrespective of the angle of presentation and the size of the test object. This points to an absence of changes in summation in the second stage of dark adaptation both at 2°50′ and 40°.

A different picture is to be seen on the left of the curves, cone vision. A considerable fall in the thresholds is observed with the small test objects, Nos. 2 and 3, at 2°50′. One must assume from the increase in light sensitivity with a decrease in the size of test object that in this portion of the retina small changes appear in the cone receptive fields. At the same time with an angular separation of 40° the curves plotted for the various test objects virtually coincide with each other and with the curve for test object No. 1 at 2°50′ (there is a certain sag in the curve of test object No. 2; it is regular and will be discussed below). We can thus state that at the periphery, at an angle of 40°, no significant changes in the receptive field are observed in the course of light adaptation.

VOL. 5 1960

These conclusions are confirmed by treating the experimental results in the following way.

Let us interpolate the arbitrary coefficient of summation $n$ as the measure of nerve integration (summation) in the retina. It may be computed from the curves of dark adaptation by means of the formula

$$n = \frac{\log \frac{I_1}{I_{2(3)}}}{\log \frac{S_1}{S_{2(3)}}}$$

where the subscripts correspond to the number of the test object.

By a somewhat different method but proceeding from the same considerations, Arden and Weale calculated $n$ [8]. It must be stated that in the work of Arden and Weale, as in our case, the calculation of summation involved a high degree of error, since to make an accurate appraisal of the changes in summation two points are insufficient. Therefore, the changes in $n$ in the course of dark adaptation in Figs. 3 and 4 reflect only the qualitative and not the absolute quantitative changes in summation.

Fig. 3 shows the changes in summation in the course of dark adaptation with the largest and smallest test objects (Nos. 1 and 3) as a function of the time of application of the light stimulus. The curves in the figure confirm the earlier conclusion that there is an increased summation in the cone phase of adaptation at an excentricity of 2°50′ and absence of changes in summation at 40°. However, this still does not demonstrate

that the nervous factor in dark adaptation consists solely in an initial decrease in the cone receptive fields with subsequent transition to unchanging rod fields.

Fig. 4 shows the changes in the coefficient of summation as a function of the time of adaptation on comparing test objects Nos. 1 and 2. It is clear from the figure that in the rod part of the curves (after the inflexion in the curves) changes in summation are present. In addition, changes in summation are found with excentricity of angle at 40°. This could not be demonstrated for test objects Nos. 1 and 3 which greatly differed in size. We would again recall here that direct quantitative comparison of the coefficients of summations in Figs. 3 and 4 is unreal and that the actual changes in $n$ in the cone part of the curves are considerably greater than those in the rods (Figs. 1 and 2).

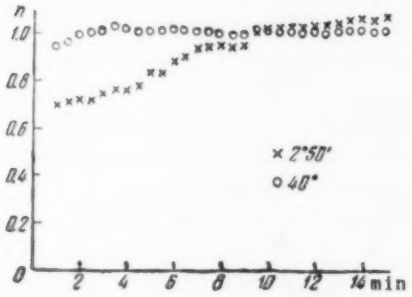


FIG. 3. Variation of summation coefficient $n$ with time of dark adaptation. Coefficient calculated for test objects Nos. 1 and 3.

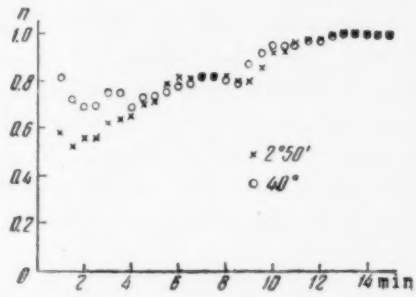


FIG. 4. Variation of summation coefficient $n$ with time of dark adaptation. Coefficient calculated for test objects Nos. 1 and 2.

Thus, the nervous factor in dark adaptation is related to changes in the size not only of the cone but also of the rod fields. At the same time it cannot be stated from our results that certain changes in summation in the region of the retina 40° distant from the centre are associated with changes only in the rod fields since large cone fields may also be operative here. However, comparison of our data with the results of Mandelbaum and Sloan [12] who found that starting from 25° distant from the centre, the minor changes in visual acuity were due only to the rod apparatus, makes this first assumption very plausible.

## SUMMARY

From an analysis of the experimental data it may be concluded that our initial premises were correct.

In addition to the adaptational changes in light sensitivity connected, according to Rushton and Cohen [1], with changes in the quantum threshold, there occur changes in sensitivity due to changes in spatial summation.

The considerable changes in summation in the course of dark adaptation are connected with: (1) changes in the size of the cone receptive fields; (2) transition from the active cone field to the rod field; and (3) minor changes in the rod fields.

They are observed in that region of the retina (3° from the centre) where visual acuity varies considerably with the adaptational background because of the separate functioning of the rod and cone mechanisms.

In the region of the retina away from the centre (40°) where the changes in visual acuity in different conditions of adaptation are not large and are connected only with the rod apparatus, the changes in summation in the course of dark adaptation are also small. It may be assumed that they are due to certain insignificant changes in the rod fields.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# PERCEPTION OF IMAGES OF VARIABLE BRIGHTNESS FIXED WITH RESPECT TO THE RETINA OF THE EYE*

A. L. IARBUS

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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IT is known that within a constant test field fixed relative to the retina, all visible differences disappear after a few seconds. The so-called empty field appears [1, 2].

In the present work we attempted to explain what are the minimal changes in the effective light at which the subject starts to see differences in the test field which remains constantly fixed with respect to the retina.

## METHODS

The test field was rendered motionless with respect to the retina by means of the suction cap depicted schematically in Fig. 1. The design of the apparatus depicted

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differed from those previously described [1] in that it enabled us to carry out the experiments without illuminating the sclera through which in normal conditions a great deal of scattered light enters the eye.

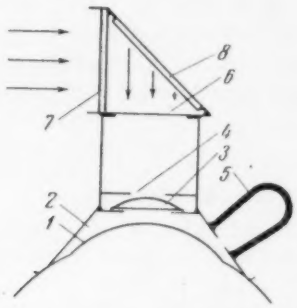


FIG. 1. Arrangement of suction disk. 1, cornea; 2, duralumin frame with crimped edges to prevent movement over the sclera; 3, lens; 4, diaphragm (artificial pupil, diameter 1·5 mm); 5, hollow rubber bulb; 6, location of test field; 7, opal glass; 8, mirror.

The rubber bulb serves to reduce the pressure in the space between the anaesthetized surface of the eyeball and the frame of the suction cap. The external pressure fixes the suction cap to the eye. The lens gives a sharp and enlarged image of the test field. The side beam of light falls only on the opal glass with the sclera remaining in complete darkness. The mirror changes the direction of the light rays deflecting them to the test field.

The test field was in the form of a round hole in black paper crossed by a very fine black silk thread. The subject saw the hole and the thread against a background of the opal glass of the suction cap at the angles indicated in Fig. 2. The brightness of the circular test field which the subject looked at through the 1·5 mm diaphragm of the suction cap was measured in apostilbs.

The opal glass of the suction apparatus was illuminated by a beam of light from an incandescent lamp. The brightness of the opal glass was varied linearly by a wedge placed between the light source and the suction cap.

During the experiment the second eye of the subject (not in contact with the suction apparatus) was bound with a black bandage and therefore when the empty field appeared, the subject so to speak was plunged into complete darkness — the bright circle of the test field merged with the black surround and "darkness" resulted.

By altering the velocity of movement of the wedge the experimenter could always make the necessary change in the brightness of the test field. For any initial brightness of the test field ($I_0$) it was easy to match such changes in brightness of the test field at which the subject for a certain period of time saw the test object distinctly, saw it very faintly or did not see it at all. The appearance of the test object was always as it were retarded in time by a fraction of a second or even for a period greater than a second. It first appeared as a barely noticeable circle, the brightness of which was constantly increased with time, then against this background the separate parts of the thread appeared and finally, the whole thread. The "threshold rate" was taken as

the rate of change of brightness of the test field corresponding to the stage at which differences in the test field were not perceived by the subject, i.e. the stage at which against the background of the circle he distinguished only the individual parts of the thread (the whole thread did not appear).

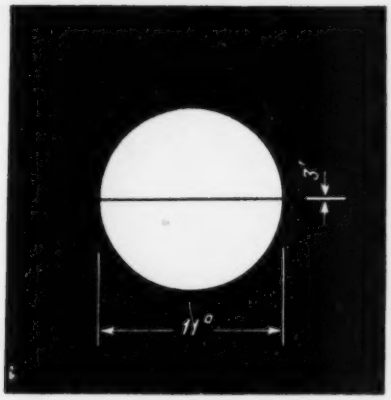


FIG. 2. Diagram and dimensions of test field seen by the subject. Diameter of light circle 11°; thickness of thread equal to 3'.

Knowing the original brightness of the test object ($I_0$), the time of movement of the wedge ($t$) and the final brightness ($I$), it was easy to determine the rate of change in brightness $dI/dt$. In fact, since the intensity varied linearly, we could write that $(I - I_0)/t = dI/dt$.

It is obvious that given an unchanged pupil the brightness at the retina ($H$) and its variations $dH/dt$ are linearly related to the corresponding brightness of the test field and the variations in the brightness.

As we have already pointed out, the differences within the test field in response to change in its brightness, were noticed by the observer not instantaneously but after a short time (fractions of a second). We shall denote this time by the letter $\tau$. As preliminary experiments showed, the value of $\tau$ is not constant and primarily depends on the value $(dH/dt)/H$. However, we did not consider this problem in detail.

Most of the measurements were made on two subjects.

## RESULTS

(1) Our main concern was to find out how the appearance of differences in the test field varied with the direction (sign) of change of intensity of this field. The experiments showed that if the test field of a certain arbitrary intensity ($I_0$) passes into an empty field, owing to its fixation relative to the retina, then for the subject it appears again both when the intensity is increased and when it is decreased. The rates of change in brightness necessary for the appearance of this test field (threshold rates) are approximately equal in their absolute value for increase or decrease in brightness.

(2) We then sought to determine whether the threshold rate of change in brightness (with constant $I_0$) depends on a variety of conditions preceding the measurement. For example, whether or not it changes as a result of the preliminary action of bright constant light, flickering light or dark adaptation. The experiments showed that at the moment of measurement, i.e. 30–40 sec after the formation of the empty field these factors had no appreciable influence on the value of the threshold rate.

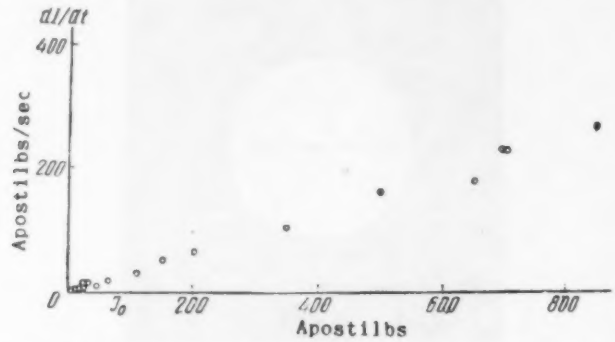


FIG. 3. Graph of relation between threshold rate of change in intensity of test field ($dI/dt$) and intensity ($I_0$) of the field.

(3) In subsequent experiments we investigated the manner in which the threshold rate of change in intensity varied with the original brightness of the test field ($I_0$). The results of the experiments are plotted in Fig. 3 which shows that within an intensity range from a few to a thousand apostilbs there is a linear relation between these values; i.e. with an increase in the intensity $I_0$ the threshold rate of change in brightness ($dI/dt$) rises proportionately.

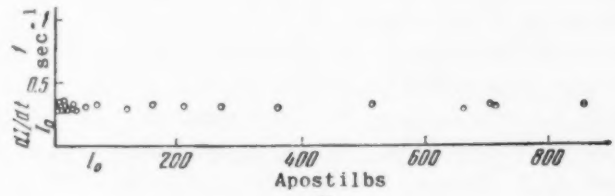


FIG. 4. Graph of relation between ratio $\frac{\frac{dI}{dt}}{I_0}$ and intensity ($I_0$) of test field. $dI/t$ is the threshold rate of change in intensity of test field.

We would note that this result is in complete agreement with the Weber-Fechner law.

(4) It follows from Fig. 3 that the ratio of the threshold rate to the value of the brightness of this field is a constant value, i.e.

$$\left| \frac{\text{threshold } \frac{dI}{dt}}{I_0} \right| = \text{const.}$$

The values of these ratios are plotted in Fig. 4. In our case these ratios, as can be seen from the graph, are approximately equal to 0·3. This means that differences in the test field fixed relative to the retina are first noticed by the subject when the brightness of this field varies by 30 per cent per sec. This figure remained unchanged for the entire range of intensities of the test field investigated.

The value of this threshold rate may appear to be in conflict with everyday experience when changes in brightness much slower than 30 per cent per sec are noticed. But this contradiction is not a real one since entirely different processes are involved here. In normal conditions of observation, because of movement of the eyes, the brightness of the individual elements of the retina is constantly changing irrespective of whether the brightness of the objects examined changes and the rate of its change. Thirty per cent per sec is the rate of change of brightness of the element of the retina at which signals arise in the corresponding nerve fibre. The changes in the illumination of objects in our experiments depend on the degree of the difference in the signals which had already appeared.

(5) It later turned out that when the ratio $|(dI/dt)/I_0| = 1$ then after a time lapse $\tau$ the subject saw the test field quite distinctly (the thread which was 3′ thick was quite distinctly visible). This ratio remained unchanged for the entire range of intensities investigated (from several to a thousand apostilbs).

If it is borne in mind that for the unchanged pupil the brightness and changes in the brightness of the retinal image bear a linear relation to the intensity and changes in intensity of the test field and that for the entire investigated range of intensities, differences in the test field were already well visible to the subject at $(dI/dt)/I_0 = (dH/dt)/H_0 = 1$; and that $(dH/dt)/H = dlnH/dt$ then the result obtained can be written in the form of the expression $dlnH/dt = 1$.

## SUMMARY

When at the retina of the eye the absolute value of the luminance, derived from the natural logarithm, becomes in time and remains greater than unity (i.e. $dlnH/dt > 1$), then after a certain time interval $\tau$ the subject distinctly sees the given test field which is fixed relative to the retina (Fig. 2). It is natural to suppose that this expression holds good for any test field. However, it may turn out that unity will be replaced by a different numerical value.

For a useful discussion of the work the author would like to express his deep gratitude to N. D. Niubyerg, Head of the Laboratory of Biophysics of Vision and to the staff of the Laboratory.

*Translated by* A. Crozy

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* There is a translation into English of this work.

# CONDITIONED FIXATION REFLEXES AND CERTAIN ASPECTS OF THE CYBERNETICS OF EYE MOVEMENTS IN MAN*

B. Kh. Gurevich

Pavlov Institute of Physiology, U.S.S.R. Academy of Sciences, Leningrad

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VOL
5
196

In setting themselves in the direction of biologically important objects, the eyes of man can rotate through considerable angles (up to 40–50°). The fixation movements of the eyes, co-ordinating the work of the periphery and the centre of the retina, form the physiological bases of the visual-spatial experience of man [1, 2]. They obviously contain links producing in the higher nervous centres compensatory effects for the movements of the eyes in such a way that displacement of images on the retina is not accompanied by sensations of movement of the environment.

Thus, with fixation movements information on the displacements of the optical axes apparently flows into the higher centres, allowing utilization of the increased possibilities of analysis of the environment established by evolution in the fovea centralis with its rich neural junctions.

It appears obvious that the proprioception of the extrinsic ocular muscles must be the direct source of information on rotations of the eyes [3]. However, it is known that there are cases (in paralysis of extrinsic ocular muscles, on misleading the eye with the finger or prismatic glasses [1], on prolonged fixation of a point [4] and vestibular nystagmus [5]), when the estimation of the direction of gaze does not agree with the actual position of the eyes. According to the most widely supported "innervation" theory [1] and its modern variants [6–8] "we know which volitional impulses and the intensity which we must apply to bring the eye to the given position".

The latter concept can be regarded as the concept of "co-ordinated efferentation" and can be put in the category of hypotheses on the "efferent" control of motor reactions [9]. There is no experimental evidence in support of such hypotheses.

The morphology and neurology of the oculomotor system do not shed any light on the mechanisms of regulation of fixation reflexes. It is common to make a theoretical division of the fixation rotations of the eyes into those controlled by the occipital centres of the cerebral cortex ("automatic" or "reflex" elicited by optical stimulation) and those mediated by the frontal cortical oculomotor centres ("voluntary" or "command" [10]), which still leaves incomprehensible the utilization of visual-motor ex-

* Biofizika **5**: No. 2, 162–169, 1960.

perience in rotations of the eyes to a non-optical signal. The neural junctions of the recently-described proprioceptors of the extrinsic ocular muscles [11] have been investigated only up to the level of the mid-brain and it can only be assumed that the higher centres of proprioception of the ocular muscles are associated with definite parieto-temporal-occipital zones of the cortex of the large hemispheres [12].

## 1. MOTOR INNERVATION AND PROGRAMMING IN "VISUAL" FIXATION

Certain indications can be emphasized in the characterization of the movements of the eyes with change of the visible objects of fixation. The eyes move in fractional jerks alternated with stops. The range of the angular velocities of these movements (from, for example, 140°/sec for rotations through an angle $\alpha = 5°$ to 400°/sec for $\alpha = 35°$ [13–15]) is close to the limit of visual perception of fast movements [16], and the duration of the rotations of the eyes is shorter than the total duration of the retinal and central-nervous reactions to optical stimulation [17]. Therefore, and also on the basis of the findings on arrests of the eyes during reading [18] and inspection of figures [19], it is considered that change in perception comes about not during the jerks but during the fixation stops. The supporters of the theory of "co-ordinated efferentation" are compelled to assume that the jerks of the eyes are "predetermined" during the stops by the "sensation of innervation", i.e. by developing programmes by virtue of which the uniform rotations of the eyes ought to have a single course. However, the divergent data in the literature [14, 15, 20] and the results of our systematic investigations [21] are not in accord with such assumptions: the mechanisms of the motor innervation and the mechanisms of programming the displacement of the eyes do to a certain extent function separately.

## 2. CONDITIONED FIXATION REFLEXES AND MECHANIZATION OF REGULATION AND CONTROL OF OCULAR MOVEMENTS

In our previous papers [21–23] we reported some results of a systematic investigation of the conditioned reflexes of fixation in man with objective (electro-oculographic) recording and statistical treatment. The reader will find details of the methods in these papers.

With conditioned reflex fixation, man in response to a non-optical conditioned signal reproduces in the dark rotation of the eyes in the definite set direction in which the visible object had previously been fixed. A direct effect of optical stimuli on conditioned reflex fixation is excluded — in view of the response which is controlled by the centres of efferentation and afferentation (direct communication and feedback) of the oculomotor system and, in addition, by utilizing the tuning mechanisms involved in "visual" fixation.

It has been shown that irrespective of the set direction, the conditioned reflex rotations of the eyes are formed as a result the combination of one to three of signals with rotation of the eyes in this direction to the visible object (Fig. 1). "Change" in the direction of fixation to the conditioned signal occurred just as rapidly. Obviously, clear vision of the object in the given direction serves as reinforcement.

Thus, in response to the conditioned signal definite system shifts are produced in the centre of the oculomotor system, which also occur in these centres on "visual" fixation — suitable adjustment is produced.

It has also been demonstrated that the position of the eyes, once attained, may persist in man in the dark for quite a long time (Fig. 2) and with considerable "spontaneous" deviations, in the form of "drift", the nerve centres may abruptly return the eyes to the given position. Drift of the eye and the regulatory jerks constitute in this case nystagmus-like movements of the eyes on both sides of a given position.

Consequently, besides the tuning mechanism, determining the final setting of the eyes in the given direction, there is at work a mechanism converting information on the deviation of the eye from the given position into effector signals.

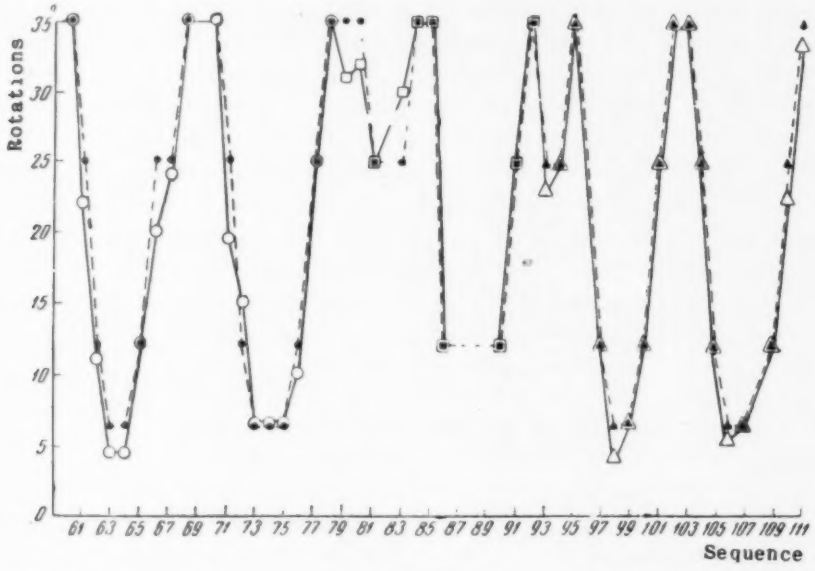


Fig. 1. Angular dimensions of conditioned fixation rotations in the dark after three-fold reinforcement by presentation of a point in a given direction.
——·—·— change in instructions; ——— changes in dimensions of conditioned fixation rotations in the dark; ●, ■, ▲ horizontal, vertical and diagonal (45°) instructions; ○, □, △ corresponding fixation rotations in the dark in the same planes. Subject N.A.F. 26 December 1957–22 March 1958.

These facts are a serious challenge to the "innervation" theory or the theory of "co-ordinated efferentation" for by keeping the eyes in a definite position the tuning and conversion mechanisms operate independently of the impulses applied to transfer the eye to the given position [24].

It was also found that the conditioned reflex rotations of the eyes in any set direction may (in about 40 per cent of the cases) be accomplished by setting the eyes in this direction and keeping them in the position reached with the same accuracy possessed

by rotations of the eye to the visible object (see Table 1): the tuning and conversion mechanisms have sufficient accuracy to play an important rôle in the control of movement of the eyes both to a non-optical and to an optical signal.

TABLE 1. NUMBER OF CASES OF ACCURATE CONDITIONED REFLEX FIXATION IN THE DARK IN INVESTIGATIONS ON SIX SUBJECTS

| Plane of rotation of eyes | Angle of instruction | Number of rotations of eyes in dark | With error of less than 30′ | | Note |
|---|---|---|---|---|---|
| | | | Number | % | |
| Horizontal | 6°20′ | 608 | 310 | 51·0 | Participation of six subjects |
| | 12° | 642 | 290 | 45·2 | |
| | 25° | 583 | 255 | 43·8 | |
| | 35° | 518 | 211 | 40·8 | |
| Vertical | Total | 2351 | 1066 | 45·3 | Three subjects participated |
| | 6°20′ | 99 | 60 | 60·7 | |
| | 12° | 176 | 67 | 38·0 | |
| | 25° | 178 | 39 | 21·9 | |
| | 35° | 232 | 54 | 23·2 | |
| Diagonal 45° between horizontal and vertical | Total | 685 | 220 | 32·1 | Two subjects |
| | 6°20′ | 134 | 69 | 51·5 | |
| | 12° | 159 | 73 | 46·0 | |
| | 25° | 198 | 64 | 32·2 | |
| | 35° | 169 | 65 | 38·4 | |
| | Total | 660 | 271 | 41·2 | |
| | Sum total | 3696 | 1557 | 42·2 | |

## 3. OCULOMOTOR APPARATUS AS A SYSTEM OF AUTOMATIC REGULATION

It has already been pointed out that the theory of "co-ordinated efferentation" requires that the composition of movements, for the same directed rotation of the eyes should be constant. Using this criterion, the theory can be subjected to a conclusive experimental check.

Let us turn to the diagram (Fig. 3) which demonstrates the fundamental scatter — which we found for all the subjects — in the composition of the conditioned reflex rotations of the eyes to any given position for all parameters, namely: (a) in the number of jerks in the rotation (for example, from one to three with a maximum of six); (b) in their dimensions (for example, from 30 to 100 per cent of the entire rotation with a minimum of 15 per cent); (c) in the duration of the individual fixation arrests (for example, from 0·04 to 0·7 sec with a maximum of 1·92 sec) and (d) in the angular velocity for equal jerks (for example, $\pm 40$ per cent with a maximum of about $+100$ per cent). In addition, in rotations of the same magnitude the order of sequence of the larger and smaller jerks may vary and cases of excessive jerks with subsequent return of the eyes to the given position are met with. Similarly, the duration of retention of the eyes in the given position in the dark may also vary appreciably.

These findings together with the material presented above demand rejection of the theory of "co-ordinated efferentation" and of the "innervation" theory as applied to fixation reflexes of movement of the eyes in fixation rotations not "predetermined" (not co-ordinated).

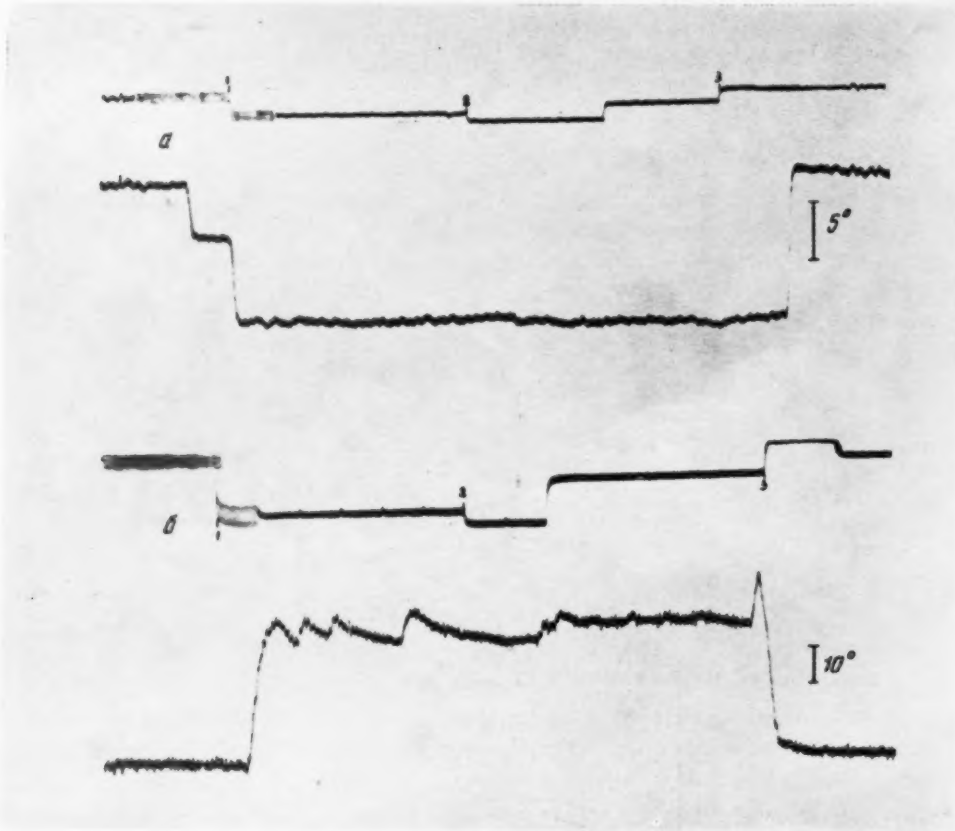


Fig. 2. Rotations and retention of eyes in definite position in the dark in response to conditioned signal. Top line, tracing of stimulations; bottom line, electrooculogram. Thickened part of trace corresponds to conditioned auditory signal. *1*, exclusion of central point of fixation (darkness); *2*, inclusion (for control and reinforcement) of point in given direction; *3*, exclusion of this point and of central fixation point. (*a*) rotations of eye in darkness horizontally; (*b*) vertically. Duration 0·2 sec.

It may be objected that in the nervous system it is not the entire composition of the fixation rotation which is predetermined but only its end "pattern". However, the errors in the first jerks for equal rotations are apparently the result of changes in the initial functional state of the centres of efferentation. In these conditions the hypothetical computer-control centre incorporated in the circuit of recurrent collaterals [25] and the insertion neurones forming a feedback to the efferent system (Fig. 4 (a))

would be faced with the insoluble problem of compensating for the errors in the various impulse flows at the different interrelated levels of the nervous system independently of the variations in the initial conditions throughout this system.

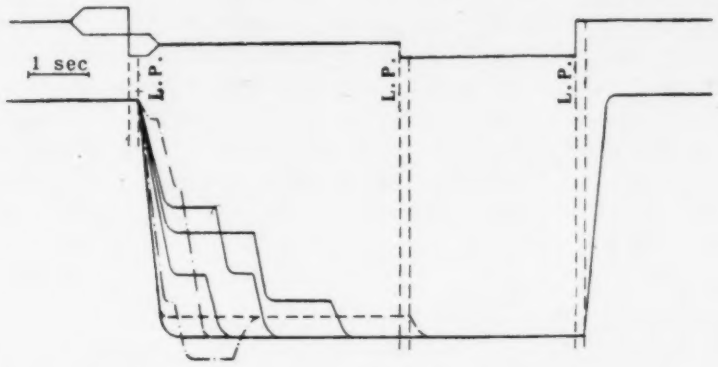


FIG. 3. Variation in characteristics of equally-directed accurate (————) or inaccurate (--------) conditioned reflex fixation rotations of eyes in repeat tests (schematic). Continuous broken line, usual changes in composition of rotation; dash-dot line, less frequently found changes. From the results of more than 2000 records of horizontal eye rotations in the dark in different set directions for six subjects. Abscissa, time of passage of conditioned response; ordinates, movement of eyes (in arbitory units). Significance of falls and final rise of the tracing of stimulation and also extension of line as in Fig. 2.

It might also be assumed that any "computer centre" compares at a definite level the frequency of the efferent impulses with that required for the given fixation and that during the arrest it converts the signals of error into effector signals. But such

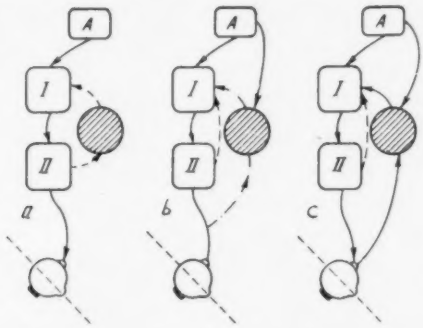


FIG. 4. Hypothetical schemes of regulation of conditioned fixation reflex rotations of eyes in the human nervous system. (*A*) central representation of conditioned signal, *I*, *II*, cortical and subcortical efferent centres; shaded circle, hypothetical "computer" centre; *a*, scheme of regulation with the "computer centre" which is not in agreement with experimental findings on the return pathways of efferent system; *b*, scheme of control of peripheral efferent impulsation with "computer centre" — not in agreement with morphological data at a higher level; *c*, proprioceptor regulation in agreement with data of a scheme involving the participation of higher centre of proprioception. Details in text.

a system of control could only operate if impulses arrived at the "computer centre", located at a sufficiently high level of the nervous system, from an outer link, i.e. from a peripheral axon innervating the muscle (Fig. 4 (b) see also [5]). Findings on the morphology of the oculomotor pathways do not provide a basis for such a hypothesis. The absence of cortical control of the frequency of impulses in the efferent fibres of the third, fourth and sixth cranial nerves is biologically understandable; such a system of control would duplicate the existing system of signalling from the proprioceptors of the extrinsic ocular muscles [11, 26], without allowing, however, account to be taken of changes in the functional state of the muscles. The proprioceptors of the extrinsic ocular muscles are the only sensory elements continuing to act during rotation of the eyes in the dark. Furthermore, the impulses from the proprioceptors have no importance as independent regulatory factors: one and the same conditioned signal, depending on the direction of the "visual" fixation with which it was earlier combined, induces rotations and setting of the eyes in different directions. The final state of contraction of the extrinsic ocular muscle is dependent on the time relations of the conditioned stimulus, i.e. on tuning of the system, and the impulses from the proprioceptors of the extrinsic ocular muscles take part in the regulation of these muscular contractions. One cannot avoid the assumption, in line with the Pavlov theory of central feedbacks [27, 28], that the conditioned signal produces temporary system shifts in the higher centres of proprioception. With the appearance of these shifts the entire oculomotor system acts as a whole as a system of automatic regulation and control of the movements of the eyes.

Fig. 4 (c) schematically depicts a hypothetical system of control of conditioned fixation reflexes in man. The conditioned signal is simultaneously the actuating one and the signal of tuning via the cortical centres of proprioception [27–29]. The initial imprecise movement of the eyes rapidly ceases and on change in the position of the eye signals from the proprioceptors of the extrinsic ocular muscles are sent to the higher proprioceptive centres. The fixation pause is occupied by the action of the conversion mechanisms; signals on the change in the position of the eyes are translated in the higher centres of proprioception into effector signals and start to act, along the association junctions of these centres, on the effector centres for the oculomotor reactions which act as a regulatory organ. The changes in the rapid, imprecise jerks of the eyes and the long periods of translating the signals of error into effector signals are repeated in cycles; the initial discord is increasingly eliminated and the fractional jerks diminished until the fixation turn reaches the given value with sufficient precision (Fig. 3), in other words, until in the higher centres of proprioception the pattern of excitation is produced which was earlier combined with the flow into the cortex of impulses from the central region of the retina giving clear vision of the object in the given direction.

Such a hypothesis of "sensory tuning" also seems to give the best natural explanation of the origin of optical illusions in cases of dissociation between the true position of the eyes and its assessment which was discussed above.

VOL
5
196

## 4. DISCUSSION ON THE GENERAL LEVEL OF PHYSIOLOGICAL CYBERNETICS

The movements of the eyes on fixation (especially horizontally) are ensured by simple neuro-muscular devices and junctions and are characterized by constancy of the mechanical load. Therefore, the conditioned fixation reflex may be regarded as model of a conditioned motor reflex.

It is important to note that in "sensory" tuning, states of the organism are reproduced which are directly connected with the moment of reinforcement [29]; this tuning is not achieved in the elaboration of the conditioned reflex, but is taken over as a final product from acts of unconditioned reflex behaviour. The effect which has to be achieved by the developing conditioned reflex is determined during the first combination of the conditioned signal and the reinforcing agent. In the formation of a conditioned reaction, its composition becomes more precise while remaining in principle variable, which ensures the utmost flexibility of behaviour of the organism to variations in its environment.

From this point of view the problem of modelling a conditioned reflex is one of designing an automaton which with repeated execution of the instructions to a certain degree completes the chain of its actions. This task is obviously facilitated, as compared with that of designing automata, by fixing the most effective acts in combination with a signal [30]. However, the usual simplicity of producing conditioned reflexes has its reverse side. This consists of the complexity of the central nervous processes; in particular, these processes must in response to the signal ensure definite tuning from the background of sensory "patterns" laid down in the organism by evolution and further elaborated in ontogenesis — one of the most important advantages of the brain over modern robots lies precisely in the vastness and universality of the background of ready-made tuning [31] and of organizing programmes.

In this context we can present in a new light the problem of the "formation of ideas by automata" [32]. The "sensory" adjustments fixed in time, may form organizing programmes of a higher order. Apparently our notions on the disposition of objects derive from the fact that the background stimuli produce in the higher sensory centres, chain system shifts which developed earlier on inspection of the objects in the environment. In the same way when travelling along a road [32] the shape of the road may be represented in the brain as a result of the flow of proprioceptive and retinal stimuli producing a chain of adjustments in the higher sensory centres.

The advantage of the formation of ideas by this principle as compared with the principle of the fixing of invariants of effective actions is obvious if in the automaton new "sensory" adjustments are formed from the elements already laid down in the long memory.

The Pavlovian principle of the formation of temporary links between the representation of the conditioned signal and the higher sensory centres of the reaction demonstrated by us in relation to conditioned fixation reflexes seems to open up definite prospects in the field of physiological cybernetics and the modelling of cerebral functions.

*Translated by* A. CROZY

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VOL
5
196

# EFFECT OF IONIZING RADIATION ON THE LUMINOUS BACTERIA *B. ISSATCHENKOI**

**(Radiobiological Problem of the Time Factor and of Recovery)**

A. A. ZOTIKOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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OL.
5
960

LUMINOUS bacteria, as Harvey [1] has shown, are a convenient and suitable object for radiobiological investigation. In fact, the glow of bacteria is a visible quantitative indication of the rate of the intracellular of oxidation of luciferin catalysed by the enzyme luciferase [2]. Luminous bacteria are one of the few biological objects in which it is possible to follow the behaviour of enzymatic systems in the living cell, both in the presence and absence of various external factors [3] including ionizing radiation.

Recently, Hug and Wolf [4] developed a mathematical theory of "the biological time factor" based on the kinetics of open systems [5] and using the concepts of rapid metabolism in the organism (the results of experiments with radioactive tracers) and the processes of recovery after irradiation (cumulative theory of the time factor) [6].

According to this theory, on irradiation of open systems at a constant dose rate a new state of mobile equilibrium is formed (at a lower level than the original one), on cessation of radiation the former state of the open system is restored.

The experiments carried out by these workers on the irradiation of luminous bacteria, strictly speaking, cannot be regarded as confirmation of the theory they developed, since the establishment of a new state of mobile equilibrium during irradiation was not observed, although recovery of the activity of the light-emitting enzyme system after irradiation was observed. The above-mentioned work [4] formed the starting-point for our investigation. However, our object was not to submit this theory to a detailed test, but merely to consider the problems relating to the "time factor" and "recovery".

## METHODS AND MATERIAL

The test object was the luminous bacteria *B. issatchenkoi* kindly placed at our disposal by A. A. Yegorova who was the first to isolate this bacteria from the waters of the Black Sea and to name it after Academician B. L. Isachenko [7].

The bacteria were cultivated on nutrient medium of the following composition†:

* Biofizika **5**: No. 2, 170–175, 1960.

† The medium was proposed by A. A. Yegorova.

Fish (pike perch) aqueous extract 500 ml

| | | | |
|---|---|---|---|
| Water | 500 ml | Aspargin | 5 g |
| NaCl | 30 g | $K_2HPO_4$ | 1 g |
| Peptone | 10 g | $MgSO_4$ | 0·5 g |
| | | Agar-agar | 20 g |

This medium differs from the usual one for luminous bacteria in that it contains a fish extract to prevent the rapid decay of luminescence. The medium was sterilized by Koch's method [8] and poured into sterilized Petri dishes. The bacteria, plated on agar, were cultured in an incubator at a temperature of 20°C for 24 hr. The colonies were sub-cultured every day. After constant luminescence was achieved, within about 2 hr, the bacteria were irradiated. The source of radiation was a BF-70 X-ray tube with a beryllium window operated as a halfwave arrangement (voltage 60 $kV_{max}$, filter 0·25 mm Al). The intensity of the light emitted by the bacteria was measured by means of a specially constructed photometer consisting of a FEU-19 photomultiplier and a M-91/A galvanometer. The photomultiplier and the object were placed in a light-tight casing. The photomultiplier was supplied with a voltage of 1200 V, from dry batteries. The dark current did not exceed $3\times10^{-8}$ A, this was 2 orders of magnitude less than the value of the photocurrent when measuring the intensity of the luminescence of the bacteria and was therefore neglected in the calculations.

The temperature during each experiment was constant at $19\pm1°$. Not less than three runs were carried out for each experiment and results were averaged.

## RESULTS

### *(1) Short and Long Term Irradiation of Luminous Bacteria*

Preliminary investigations showed that our culture of luminous bacteria was able to restore luminescence after the action of transient X-irradiation. Therefore, it was of interest to carry out experiments on the "time factor" with a wide range of dose rates. Short and protracted irradiation of luminous bacteria was carried out with the same total dose. The difference in dose rate was a 45-fold.

In the first series the transient irradiation lasted 2 min and the protracted 1·5 hr. The uniformity of the dose (80 kr) in both cases was checked by a special dosimeter.

The intensity of the bacterial luminescence was measured before and after irradiation. The results of these experiments are shown in Fig. 1.

In the second experimental series the prolonged irradiation lasted 15 min and the transient for 20 sec. Unfiltered radiation was used. The total dose in this series was 100 kr. The results of the experiments are given in Fig. 2.

As Figs. 1 and 2 show, protracted irradiation depressed the luminescence about twice as strongly as did the transient. Thus, "an inverse time factor" was detected.

The intensity of irradiation in this case apparently influenced the break in the chain of developing reactions.

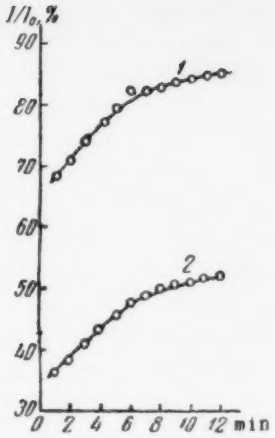


FIG. 1. Effect of transient (1) and prolonged (2) X-irradiation on luminescence of bacteria. Dose 80 kr. Abscissa, time after irradiation; ordinates, intensity of luminescence.

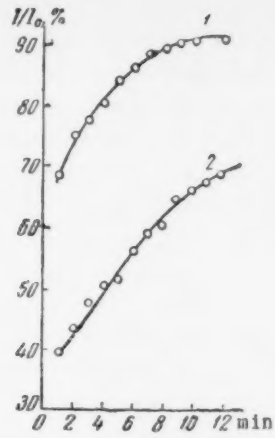


FIG. 2. Effect of transient (300 kr/min) (1) and prolonged (2) X-irradiation on luminescence of bacteria. Dose 100 kr. Symbols as in Fig. 1.

### *(2) Recovery of Luminescence*

It is clear from the curves depicted in Figs. 1 and 2 and also from the paper [4] that after irradiation there is a partial recovery of the luminescence of the bacteria. In view of the theoretical and practical importance of the fact of a recovery of the functions of the biological system after the action of ionizing radiation, we carried out special experiments on luminous bacteria devoted to this problem.

The method described above was used. The bacteria were irradiated with a dose of 160 kr at a dose rate of 40 kr/min. Several runs were made and the results of the experiments are given in Fig. 3.

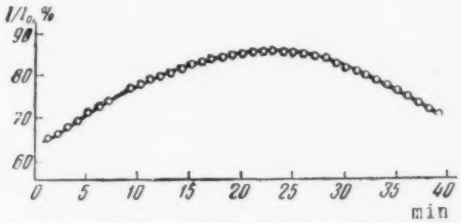


FIG. 3. Change in luminescence of bacteria after X-irradiation. Symbols as in Fig. 1.

It is clear from Fig. 3, that at first there was a rise in the intensity of luminescence of about 20 per cent which indicates recovery of the bacterial luminescence. After 18–20 min, constant luminescence was achieved. However, it did not last long, 6–8 min. After this, irreversible quenching of luminescence began and after roughly 100 min the intensity of luminescence was 10–12 per cent of the original value ($I_0$). Complete cessation of luminescence came about after 3–4 hr. Transfer to fresh nutrient medium

immediately after irradiation did not lead to visible multiplication of the cells nor consequently, to visible luminescence of the bacteria. As will be shown below, the doses used were too high to leave unimpaired the ability of the cells to multiply. Thus, there is scarcely any sense in speaking here of the recovery of the function of the enzymatic light-emitting system. There was in fact, a certain burst of luminescence but its mechanism is not clear. Apparently, 20–25 min after irradiation with such large doses "complete autolysis of the cell" begins, leading to death.

We would point out that quenching of luminescence after irradiation was not described in the paper by Hug and Wolf [4] although the doses they used were also quite high ($\sim$ 300 kr). On the basis of our experiments we must conclude that the recovery of the luminescence of the bacteria they observed was deceptive and transitory. Nor has the theory of recovery put forward by Hug and Wolf been confirmed in other radiobiological investigations [9].

### (3) *Dose-Survival Curves*

The overwhelming majority of irradiated bacteria show an exponential survival rate curve [6, 10, 14]. However, a number of workers have observed deviations from the exponential [11, 12]. Therefore, it was of interest to record the dose-survival curve for our luminous bacteria.

A modified technique was used by combining the methods described in the two papers [11, 14]. Two series of experiments were carried out. The bacteria were irradiated in suspension. The constancy of concentration of the suspension was checked by a FEK-M photocolorimeter with a blue light filter. In all cases the source of radiation was the X-ray tube with a beryllium window described above. All the experiments were carried out at least three times.

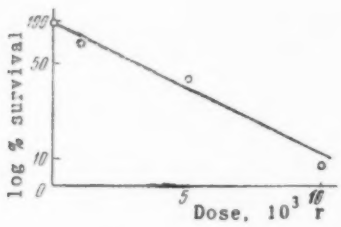


FIG. 4. Survival of bacteria as a function of the dose of X-irradiation.

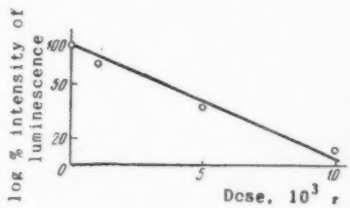


FIG. 5. Dose-"luminescence" curve of colonies of surviving bacteria.

In the first experimental series the survival rate of bacteria was determined as a function of dose of X-irradiation by the usual method of colony count. The luminous bacteria were cultivated for 24 hr on an agar slope in a tube at a temperature of 20° and then transferred to the suspension. Normal sterile water was used since the optimal value of pH for the luminescence of the bacteria lies in the region close to 7 [13]. After this the bacterial suspension was irradiated with different doses. Since for the bacterial count the transparency of the medium is essential, the previous medium

had to be abandoned. Conventional meat-peptone agar was used with the addition of 3% NaCl. It was first established that the luminous bacteria grew well and luminesced in this medium. Since our culture was susceptible to cold, special attention was paid to the temperature of the agar during the pouring of the bacterial suspension. The culture was incubated for 96 hr at a temperature of 26° and not for 48 hr as in the case of *Achromobacter fischeri* [14]. The colony count was then made. The results of the experiments are given in Fig. 4. As this figure shows the dose-survival curve had an exponential form. The dose for a 50 per cent inhibition of multiplication was equal to about 3 kr.

The same technique was used in a second series of experiments, the difference being that the irradiated culture was incubated for 24 hr at a temperature of 22°. The intensity of luminescence was measured at the end of this period by means of the photomultiplier and galvanometer. The results, as a percentage of the controls, are shown in Fig. 5. In this case also, as is clear from Fig. 5, the dose curve is of an exponential nature. This is not surprising since it is the intensity of luminescence of the bacteria capable of multiplication and producing colonies that was measured. The dose depressing luminescence by 50 per cent was 3500 r, which, within 20 per cent, coincides with the LD50 value determined by the method of colony count.

VOL. 5 1960

An interesting feature was that the LD50 for our luminous bacteria ($\sim$ 3 kr) was commensurate with the value of the LD50 for the majority of other bacteria. Therefore, it may be assumed that the mechanism of radiation death in all bacteria is similar.

However, the enzymatic processes (luminescence, respiration, etc.) were quite stable to the action of ionizing radiation. Thus, in separate experiments we established that doses of the order of 600 kr at high dose rates depressed the activity of the light-emitting enzymatic systems by 50 per cent, and after irradiation with huge doses (of the order of $1{\cdot}5 \times 10^6$ r) the intensity of luminescence of the bacteria was still 15–20 per cent of the original value. In the experiments of Meisel' *et al.* [17] it was shown that after massive doses ($\sim 10^6$ r) the respiration of micro-organisms (yeasts) was 40–50 per cent of the original value.

Therefore, study of the behaviour of enzymatic systems *in vivo* both during and after irradiation is of considerable radiobiological interest. Further experiments on the radiation of luminous bacteria *in vivo* and also with isolated systems of luciferin-luciferase will, without doubt, help to elucidate the mechanism of the biological action of radiation and explain our results.

## SUMMARY

(1) The results of experiments with X-irradiation of the luminous bacteria *B. issatchenkoi* showed that after irradiation with doses of the order of 100–200 kr, there occurs a partial recovery of activity of the light-emitting enzymatic luciferin-luciferase system. However, this recovery is temporary and transitory.

(2) The results of experiments with transient and prolonged radiation of luminous bacteria, the ratio of dose rates was 1 : 45, showed that protracted irradiation de-

presses the luminescence about twice as strongly as transient irradiation, so that an "inverse time factor" is present.

(3) The dose-effect curve was plotted by the usual method of colony count. The curve was exponential. The dose depressing multiplication by 50 per cent (LD50) was 3 kr.

(4) A dose — "luminescence" curve for the colonies of bacteria was plotted. The curve was exponential. The dose depressing the luminescence of the bacteria which formed colonies by 50 per cent, measured 24 hr after irradiation was 3500 r.

(5) It has been shown that doses of the order of $1{\cdot}5 \times 10^6$ r depress luminescence by 80–85 per cent of the original value of the intensity of luminescence of the bacteria when measured directly after irradiation.

In conclusion, I should like to express my thanks to Prof. Ia. L. Shekhtman for his constant interest in the work and for discussion of the results, and also to A. A. Yegorova for the cultures of luminous bacteria *B. issatchenkoi* and for consultations on microbiological problems.

*Translated by* A. Crozy

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VOL 5 196

# NUCLEIC ACID CONTENT AND RADIOSENSITIVITY OF *ESCHERICHIA COLI* *

YE. V. MOISEYENKO

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL. 5 1960

THE theory, originally developed by Lea [1], that on irradiation of micro-organisms the dose-survival curve is always of an exponential character has been shown to be false. A number of investigators, in particular, Hollaender, Stapleton and Martin [2] have shown that the form of the curve depends on the conditions of irradiation and culture of the micro-organisms and on the composition of the nutrient medium.

In the work of Shekhtman *et al.* [3] it was established that cultivation of *Escherichia coli* on a medium containing 2% glucose considerably enhances the resistance of this culture to the action of radiation and the culture obtained gives an S-shaped dose-survival curve. The most important aspect in their work is that this S-shaped dose curve is maintained not only on irradiation with X-rays but on irradiation with polonium α-rays for which the specific ionization is 80 times greater than for the X-rays used. In addition, the "glucose" culture obtained differed from the usual culture in the much greater size of the organisms.

In the work of Shekhtman *et al.* [3] the assumption was made that the S-shaped form of the curve and the high radioresistance of the glucose culture are connected with the polyploidy of the nuclear structures ("nucleoides") of the new culture of *E. coli*.

The role of cell polyploidy was demonstrated in experiments on the irradiation of cells characterized by a variable degree of ploidy [4–6].

In these studies the radiation damage, estimated from the ability to multiply, was related to damage of the nuclear components. An increase in their number was accompanied by an increased radioresistance; apparently, the functions of the damaged structures were carried out by undamaged structures.

It is interesting to compare these results and the conclusions drawn from them with the data for bacteria obtained by various workers. The qualification must be made that the very idea of the presence of a nucleus in bacterial cells and its possible genetic function have until now remained a subject of controversy amongst microbiologists. Nevertheless, it is commonly considered that the divergent structural formations of nuclear origin in bacterial cells (nucleoides) apparently perform the same function as the compact nuclei of other cells [7].

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Enhanced resistance of the large cells of *Micrococcus aureus* has been observed by Clark and Webb [8]. These large cells were obtained from a normal culture in defined conditions of cultivation or by the action of formaldehyde.

On irradiation with ultra-violet or X-rays the survival curve for normal cells was exponential, for the large cells it had an S-shaped character. The authors explain the enhanced resistance of the large cells by the appearance in them of polyploidy.

Cytological observations have shown an increased amount of nuclear material in these large cells.

A detailed investigation was carried out by Ogg and Zelle [9, 10] who studied the action of radiation on two strains of *E. coli*, one of which (strain P6) was obtained by the action of camphor fumes on the 82/r original strain.

The survival curve for cells exposed to X- and $\gamma$-rays for strain 82/r was of an exponential character whereas strain P6 was more resistance with an S-shaped survival curve. To explain these survival curves the authors resorted to the concept of the role of cell ploidy in the radiation effect. Analysis of the curves obtained from the point of view of the target theory showed that if the 82/r strain is taken as a haploid in accordance with the exponential character of the survival curve, then the P6 strain must be a diploid. The very choice of the action of camphor fumes to obtain the strain was based on the fact that, as has been shown in a number of studies, treatment with camphor leads to the appearance of giant, possibly polyploid forms in yeast, moulds and certain other organisms in which the polyploid form may be demonstrated cytologically.

The biochemical investigations carried out by Ogg and Zelle revealed that the large cells of the P6 strain had about three times as much DNA and RNA (2·75 for DNA, 2·85 for RNA), and that the dry weight per cell was also three times greater than in the original strain.

Cytological investigation showed that the average number of nucleoides per cell in the P6 was 1·5 times greater than in the 82/r strain. Since the DNA content per cell was three times greater, the authors concluded that the P6 strain is a diploid derivative of the 82/r strain. However, investigation of the rate of the enzymatic reactions, the rate of growth of the cultures and the frequency of mutation did not yield any convincing data indicating polyploidy.

The increased content of DNA and RNA, the large dimensions of the cells, the shape of the dose-survival curve and certain findings in the genetic analysis of the results of crossing different lines of *E. coli* in the work of Ogg and Zelle still remain the basic evidence for the polyploid nature of the modifications obtained. In the paper by Shekhtman *et al.* [3] the main, though inconclusive arguments in favour of polyploidy, in addition to the enlarged size of the cells, was the presence of an S-shaped form of the dose-survival curve on irradiation with polonium $\alpha$-rays.

If the assumption of the polyploid structure of the nuclear substance of a glucose culture is valid, then in the cells of the glucose culture the content of nucleic acids ought to be considerably greater than in the cells of a normal culture of *E. coli*. To

test this assumption the DNA and RNA contents in cells of both cultures were determined.

Determination of the content of the nucleic acids was made on the dry bacterial mass. The cultures were grown in flasks on the usual medium and on a medium containing 2% glucose. After 24 hr the cultures were washed off the flasks, then washed with three changes of water, three times in 2% cold $HClO_4$, three times in alcohol and once with ether, after which the samples were dried in a desiccator for 2–3 days. Separation of the RNA and DNA was made according to the method of Schmidt and Tannhauser, quoted in [11]. The amount of nucleic acids was determined spectrophotometrically by the method proposed by Spirin [12]. The cell count was made in a Goriayev counting chamber. The results obtained are given in Table 1.

Three samples of the dry bacterial mass were analysed for each culture. The table gives the mean values of three experiments. The determination showed that the cells of the glucose culture contained 2·7 times more DNA and 4·8 times more RNA than the cells in the normal culture.

The calculation of the volume of the cells of these cultures showed that the cells of the glucose culture were about five times greater in volume than the cells of the normal culture (in linear dimensions 1·7 times).*

TABLE 1. NUCLEIC ACID CONTENT OF NORMAL AND GLUCOSE CULTURES

| Nucleic acid | Content per mg dry weight, in γ | | Content per $10^9$ cells, in γ | | G/N ratio |
|---|---|---|---|---|---|
| | N | G | N | G | |
| DNA | 38 | 30 | 11 | 30 | 2·7 |
| RNA | 100 | 139 | 29 | 139 | 4·8 |

Note. N = normal culture; G = glucose culture.

The dry weight calculated per cell for the glucose and normal cultures differed by the factor of 3·5. A certain discrepancy in the increase in volume of the cells of the glucose culture (five times) and the dry weight of the cells is to be explained frst by the fact that during washing to obtain the dry bacterial mass some of the compounds were extracted from the cell and secondly by inaccuracies in the cell count. The glucose and normal cultures did not differ in water content (moisture content of the normal was 78 per cent and of the glucose culture 80 per cent).

Thus, the increased size of the cells in the glucose culture is not due to swelling but constitutes a proportional increase in the mass of the cell, the nature of which we still cannot explain.

* The ratio of the linear dimensions of the organisms in normal and glucose culture was equal not to 2·7 as indicated in the paper [3] but to 1·7.

## DISCUSSION OF RESULTS

The ability of cells to divide is very sensitive to the action of irradiation, this is associated with damage to the nucleoproteins which play a decisive role in the process of cell division [3]. The nucleoprotein structures of the nucleus are of a unique character and therefore local damage to these structures is sufficient to cause a disturbance in the whole cycle of processes associated with mitosis and multiplication.

In all the papers on bacteria quoted, [3, 8–10], the large cells, the appearance of which was due to various factors (glucose — Shekhtman *et al.*, camphor fumes — Ogg and Zelle, formaldehyde or selection of cultures — Clark and Webb) are characterized by an increased content of nuclear material. Apparently, it was not mere coincidence that in all three bacterial cell cultures the increased content of nucleic acids was accompanied by enhanced radioresistance.

The results obtained by us which showed a rise in the DNA content in the glucose culture, support the idea that this new culture is of a polyploid nature. The aim of further research will be to reveal the mechanism of formation of polyploid cultures when subjected to various factors (composition of nutrient medium, action of pharmacological and chemical agents) and to study the link between radio-sensitivity and metabolism in *E. coli*.

The author wishes to express his thanks to Prof. Ia. L. Shekhtman, who directed the work, for consultations and discussions of the results and to G. V. Filippova for assistance in calculating the linear dimensions of the cells.

VOL
5
196

## SUMMARY

Determination of the DNA and RNA content in two cultures of *E. coli* — normal and "glucose" — differing in radio-sensitivity and the shape of the dose-survival curve, showed that the DNA content per cell was 2·7 times and the RNA content 4·8 times greater in the "glucose" than in the normal culture, which may serve as the basis for assuming the cells of the "glucose" culture to be of a polyploid character. The cells of the "glucose" culture were five times greater in volume than the cells of a normal culture and 3·5 times greater in dry weight. The cultures did not differ in their water content.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# THE "AFTEREFFECT REACTION" IN IRRADIATED ERYTHROCYTES*

I. I. GITEL'ZON and I. A. TERSKOV

Institute of Physics, U.S.S.R. Academy of Sciences, Krasnoiarsk

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A SPECIFIC basic feature of the biological effect of radiations is the ability of the processes initiated by them to show subsequent self-development in the metabolic conditions of the irradiated cell or organism [1].

One of the ways of elucidating the mechanism of the biological effect of radiations is to employ those elemental living systems in which the reproduction of a self-developing process after irradiation is possible. Perhaps the simplest object of animal origin which can be subjected to radiation for this purpose is a mammalian red blood cell.

Mammalian erythrocytes do not have any nuclei, are devoid of protoplasmatic organoids and have a restricted set of enzymes. In addition, the erythrocyte is a living cell entity the metabolism of which though specialized, is sufficiently involved and regulated for constancy of cell structure to be maintained by active synthesis. These features of the erythrocyte would offer highly favourable possibilities for investigation of the radiation reaction in its simplest form if this reaction could be induced in the erythrocyte.

Despite the fact that haemolysis is one of the most favoured tests in radiobiology only one facet of the reaction of erythrocytes has by and large been studied — their immediate, or in the first hours after irradiation, destruction [2–8]. Only in the work of Frank are there any indications of the possibility of development of a more protracted process in blood cells [9].

Thus, the question as to whether it is possible in principle to initiate a process in non-nucleated erythrocytes comparable with the reaction after effects characteristic of living organisms remains open.

For these reasons we sought to examine the possibilities of producing in erythrocytes a process similar in its kinetics to the reactions developing after irradiation in more complex cells and whole organisms.

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As an indicator of the state of the erythrocytes we selected their stability to injurious radiation.

This choice was based on the following considerations: as long as the chemical substrate of the radiation reaction remains unknown, in selecting an individual biochemical indicator as a test there is a real danger that not the primary process will be measured but a secondary reaction. Stability, however, is the most general characteristic since it is determined by the degree of the structural orderliness of the cell as a whole. Another, but no less important consideration is that any system of cells including erythrocytes is by definition heterogenous both in age and condition.

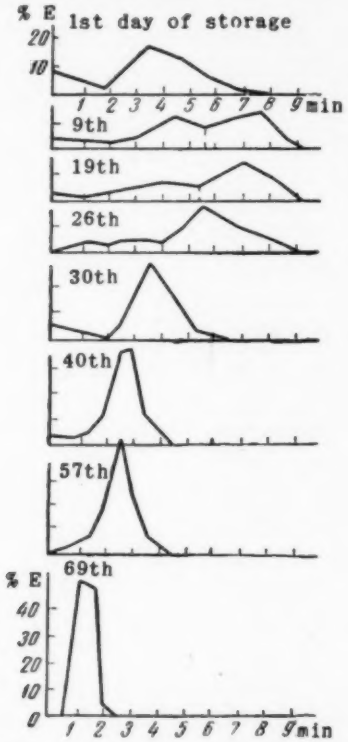


FIG. 1. Erythrograms during storage of blood preserved in formula 7. Storage temperature 20°; days on which analyses made indicated next to curves.

This heterogeneity of background must be taken into account. However, in biochemical determinations it is masked by averaging the indices for many cells.

In contrast to this a technique has been developed to characterize the stability of erythrocytes, not by an average figure, but by describing the cell distribution in terms of stability [10].

The distribution of erythrocytes in terms of stability is obtained by treating them with an accurately known dose of a haemolytic agent and by determining the sequence of involvement of the erythrocytes in haemolysis.

In our work the haemolytic agent was 0·004 N HCl. The dynamics of haemolysis were investigated by the photometric method. The curve obtained reflected the sequence of erythrocyte involvement in haemolysis and therefore it is easy to calculate from it the stability distribution of the erythrocytes. The criterion of stability for each cell was the duration of the resistance to the destructive action, i.e. the time elapsing from the introduction of the haemolytic agent up to the disintegration of the cell.

The experimental results presented below are set out in the form of graphs, known as erythrograms, with the time of the action of the haemolytic agent in minutes as abscissa and the percentage of erythrocytes as ordinates. A detailed description of the technique of erythrograms has been published earlier [10]. The aim of the present work was to investigate changes in the state (stability) of erythrocytes on prolonged storage of blood after irradiation. Irradiation was carried out with a GUT-400 cobalt unit. The doses were from 2–200 kr at a dose rate of 400 r/min. The blood of healthy donors, preserved with formula 7, was investigated. In each experiment the blood from one donor was put into two ampoules, one of which was irradiated, the second serving as control. Later, the ampoules were stored at 20°C and investigated together. The blood was withdrawn aseptically for analysis every 2–3 days.

VOL. 5 1960

The experiment was conducted against the background changes in stability developing on storage of unirradiated blood. Fig. 1 depicts erythrograms from the 1st to 69th day of storage. Analysis of the variations in stability observed in the unirradiated blood showed that they fall into three main categories:

(1) At first, the stability of all the erythrocytes increased due to the fact that the preservative made the medium hypertonic and decreased cellular permeability, a process indicated by the elongation of the erythrograms to the right. After osmotic equilibrium was established between cells and plasma these changes ceased.

(2) At the same time another process of increased stability developed differing from the first in that it did not occur simultaneously for all the erythrocytes and the changes were such that it seemed as though two discrete states of stability for the erythrocytes are possible in preserved blood; firstly, the initial state and also a state of enhanced stability. The conditions of storage promoted the passage of the cells into the second state, but this transition was not achieved simultaneously by all the erythrocytes and therefore, the blood in this period consisted of cells of two "types". This was reflected in the erythrograms in the form of a double peak distribution. The cause of this transition is not yet finally clear. It may be associated with the transition of the protoplasm of the erythrocytes into a paracrystalline state.

(3) The second half of the period of storage was characterized by a reversal of the process. The stability of the erythrocytes began to decline steadily to the zero point, i.e. to the point of disintegration of the cells without the addition of the haemolytic agent. This process is reflected in the erythrograms in the form of a gradual shortening and shift of the maximum to the left. The cause of this fall in stability was the lack of homogeneity in the age and the inhibition of the active metabolism maintaining the integrity of the cellular microstructure.

All three processes had a positive temperature coefficient.

The results reported again show that erythrocytes are living cells in which anabiosis produces regularly-developing changes on preservation.

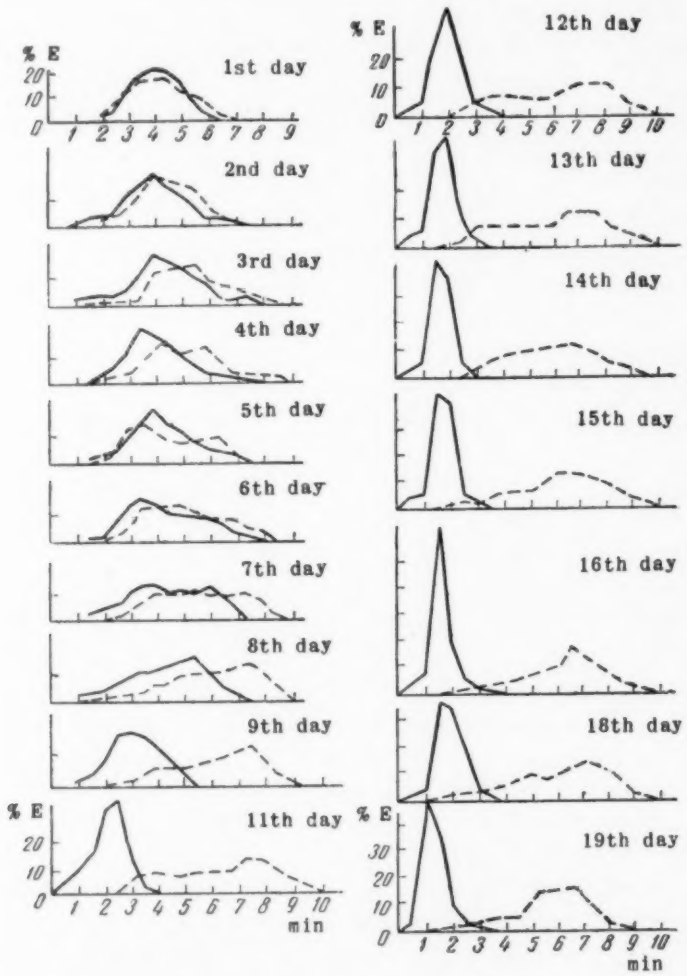


FIG. 2. Erythrograms during storage of blood irradiated with a dose of 100 kr. Storage temperature 20°; unbroken lines give erythrograms for irradiated blood; broken lines erythrograms of unirradiated blood of same donor.

Irradiation influenced the course of these processes in the following manner:

With small doses of the order of 2–4 kr the reliability of the changes observed was borderline. With doses measured in tens of kr distinct changes appeared as indicated in Fig. 2.

It is most important to note that immediately on removal from the beam the erythrocytes of the irradiated blood did not differ in stability from those of the unirradiated blood. Consequently, doses of this order did not directly damage the cell structure.

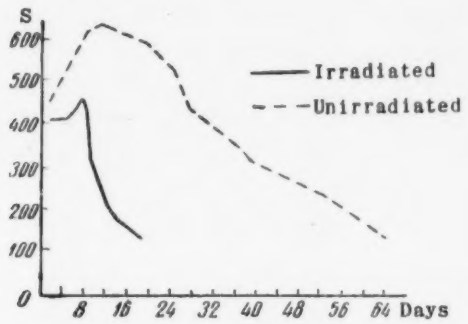


FIG. 3. Changes in overall stability of blood (S) on storage. Unbroken line, irradiated blood (100 kr); broken line, control blood.

On the 2nd day of storage a difference became apparent between the irradiated and unirradiated blood, a difference which increased with time. The fall in stability developed in the irradiated erythrocytes more rapidly than in the unirradiated ones and with time the difference in stability increased. The increasing divergence between the erythrograms can be clearly seen in Fig. 2.

Quantitatively, these differences may be represented by the changes in the overall stability ($S$) calculated from the formula:

$$S = \% E_1 t_1 + \% E_2 t_2 + \ldots\ldots + \% E_K t_K$$

where $\% E_i$ is the percentage of erythrocytes disintegrating per $i$ min, and $t$ is the time (in min) from the start of haemolysis. Fig. 3 gives graphs of the changes in the total stability for the irradiated and unirradiated blood.

Reduction in stability resulted in haemolysis. Therefore, by measuring the decline in the number of erythrocytes, in the stored blood samples, the level of the reactions leading to haemolysis may be assessed. Fig. 4 shows that in the first 12 days there was no basic change in the number of erythrocytes in both samples.

In the normal blood haemolysis then developed slowly, disintegration of 75 per cent of the erythrocytes taking 60 days; in the irradiated blood, this process starting also as from the 12th day developed within 10 days.

A comparison of the character of the changes in the erythrograms in Fig. 2 shows that they were uniform for the irradiated and unirradiated blood. In both cases, all three types of change of stability peculiar to stored blood were observed. The most characteristic feature of preserved blood was the formation of double peak erythrograms as the result of the passage of some of the erythrocytes into a state of enhanced stability — observed also in the irradiated blood. Fig. 5 presents graphs characterizing this process quantitatively. It can be seen that the transition of the irradiated erythrocytes

into a state of enhanced stability at first took a similar course to the controls but then was broken off by the incipient fall in stability.

In the irradiated, as in the unirradiated, erythrocytes initially a general increase in stability occurred which later declined. The similarity of the changes in stability in normal conditions and after irradiation is confirmed by the graphs in Fig. 3, which show the course of the overall stability.

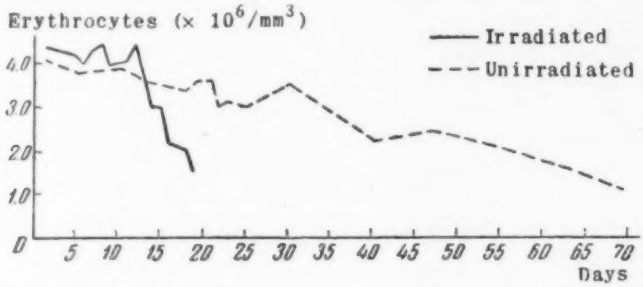


FIG. 4. Fall in number of erythrocytes on storage. Unbroken line, irradiated blood (100 kr); broken line, controls.

It is relevant to point out that the greater slope in the curve of changes in the overall stability observed at the end of the investigation cannot be considered as a sign of retardation of the process of the decline in stability. In this period the erythrocytes with the least stability were immediately haemolysed in the blood and escaped analysis. Thereby, the stability distribution shifted towards the more resistant cells. In the graph this appears as a retardation in the fall in stability.

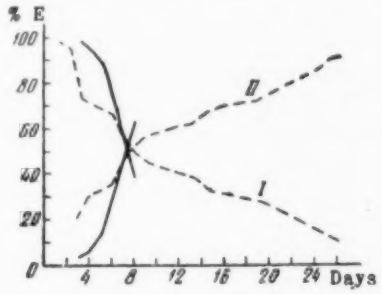


FIG. 5. Passage of erythrocytes of preserved blood into state of enhanced stability. *I*, number of erythrocytes with normal stability; *II*, number of erythrocytes in state of enhanced stability; unbroken line, irradiated blood; broken line, controls.

Consequently, in our experimental conditions no changes in stability specific for radiation damage could be demonstrated. The difference in the changes in the irradiated and unirradiated blood consisted in a higher rate of development of the processes leading to a decline in stability and the disintegration of the cell structure.

The results obtained show that in the conditions of the investigation with doses of the order of tens of kr the erythrocytes after irradiation display no appreciable

structural disturbances. But they contain within them the precursors of reactions which on subsequent incubation lead to premature breakdown of the structure and to death of the cells.

Thus, the basic pattern of radiation aftereffect observed in more complex cells and whole organisms (the self-development of a process once initiated) was detected in erythrocytes.

The development of such a process in erythrocytes which are devoid of a nucleus and protoplasmatic organoids shows that the radiation aftereffect may also develop in the absence of these structures.

Taking into account the methodological advantages of using red cells, mentioned at the beginning of the article, and the possibility of development in this elementary cell of a radiation aftereffect make it of interest to employ this object for a detailed investigation of the quantitative laws of the dynamics of development of the radiation reaction.

*Translated by* A. CROZY

VOL. 5 1960

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* There is a translation into English of this work.

# INFLUENCE OF IRRADIATED PLANT SEEDLINGS ON THE GROWTH OF UNIRRADIATED SEEDLINGS*

V. I. KOROGODIN and T. G. MAMEDOV

Faculty of Fundamental Biology of the Lomonosov State University, Moscow

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THE results reported in the literature of the influence of irradiated plants on the growth of unirradiated ones are conflicting. King, for example, reported the presence of such an influence on the cultivation of unirradiated specimens of tobacco callus next to

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irradiated ones [1]. Other workers have not been able to discover such an effect [2, 3]. It should be noted that these workers used different specimens and methods in their investigations. To elucidate the problem of the existence of an influence exerted by irradiated plants on the growth of unirradiated ones we carried out experiments with several plant species, the results of which are presented below.

## METHODS

The test objects were the seeds of cucumbers, radish (the white — pink variety), peas (Capital variety) and beans (the black Russian variety).

The cucumber, radish and pea seeds were grown in moist cottonwool.

The germinating seeds were irradiated with differing doses of X-rays from a URS-70 unit (30 kV, 15 mA). The irradiated cucumber and radish seedlings were grown next to the unirradiated ones on moist filter paper in Petri dishes and the irradiated and unirradiated pea seedlings in glass vessels of 250 ml capacity containing water (ten irradiated seedlings and ten unirradiated ones).

The bean seeds were soaked in tap water for 24 hr. On germination, the seeds were placed in moist sawdust. After 2 days the seedlings, measuring about 2 cm in length, were irradiated with different doses of $\gamma$-rays from a GUT-400 unit (dose rate 30 r/min). As "detectors" we used seedlings with a uniform rate of growth obtained by threefold selection (every 24 hr) of seedlings of the same length. The irradiated seedlings were grown in batches of 15 in vessels of 30 ml capacity containing distilled water.

The influence of the irradiated bean seedlings on the growth of the unirradiated ones was studied by three methods: (1) the unirradiated seedlings — the detectors — were placed into vessels with the irradiated ones and grown together for 5 days (in different variants of the experiment detectors of different age were used); (2) the detectors were cultivated in vessels containing distilled water into which were placed the irradiated and unirradiated seedlings cut into small fragments (about 5 mm) or homogenized by grinding down with quartz sand; (3) the redox properties of the medium in which the irradiated or unirradiated bean seedlings were grown were studied by means of an indicator, bromothymol blue, and a colorimeter, FEK-4 apparatus (three drops of standard solution of indicator per 10 ml medium; red light filter).

The seedlings were grown in an incubator at 20°. Their length was determined daily for 5–6 days. Several runs of each experiment were made.

## RESULTS

The experiments showed that joint cultivation for several days of unirradiated seedlings together with those irradiated leads to inhibition of the growth of the detectors. In the case of bean seedlings an effect could only be established on cultivation of 3-day old detectors with 5-day old irradiated seedlings. Some of the results obtained are depicted in Fig. 1.

Appreciable inhibition of growth of the detector seedlings was observed after irradiation with doses not exerting a very potent biological action (cucumbers 2000–5000 r, radish 5000 r, peas 1000–2000 r, beans 150 r). No effect could be observed

with smaller doses or doses producing a strong retardation of growth of the irradiated seedlings (Fig. 1 (4)) or causing such damage to the rootlets that they showed hardly any growth on subsequent cultivation and underwent necrosis after 3–5 days (Fig. 1 (1–3)).

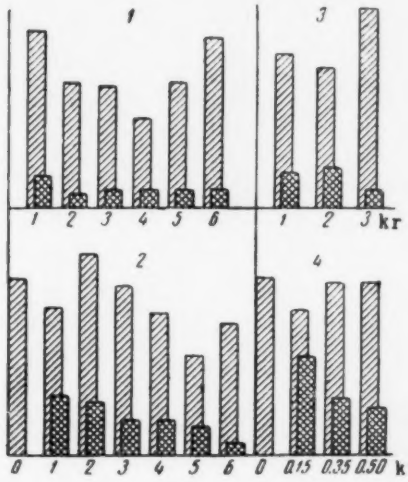


FIG. 1. Effect of seedlings irradiated with different doses on growth of unirradiated ones (final length of seedlings in relative units). Cross-hatched columns — irradiated seedlings, hatched columns — "detector" seedlings. *1*, cucumbers; *2*, radish; *3*, peas; *4*, beans.

The results of the experiments with the ground and minced bean rootlets are given in Table 1.

TABLE 1. COURSE OF GROWTH OF DETECTOR SEEDLINGS IN MILLIMETERS IN MEDIUM WITH HOMOGENIZED AND MINCED IRRADIATED ROOTLETS

| Group | Dose r | Duration of cultivation in days | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| Homo-genates | 0 | 21·1±2·4 | 51·6±4·0 | 64·0±5·3 | 83·7±5·5 | 97·1±5·2 | 109·0±5·6 |
| | 150 | 22·0±2·2 | 49·7±5·1 | 66·3±5·0 | 85·6±5·9 | 96·3±5.1 | 110·3±5·4 |
| | 350 | 21·6±2·5 | 52·3±4·9 | 63·1±4·6 | 81·4±5·1 | 95·0±5·3 | 112·0±5·7 |
| Minced | 0 | 20·3±3·1 | 50·3±4·2 | 61·9±4·9 | 81·4±5·1 | 96·1±5·2 | 105·5±5·1 |
| | 150 | 22·0±2·4 | 48·9±5·2 | 59·3±5·7 | 74·9±5·8 | 81·1±5·8 | 89·7±5·8 |
| | 350 | 21·4±2·8 | 51·0±5·5 | 63·1±5·0 | 84·0±5·5 | 88·2±5·7 | 104·9±5·9 |

An inhibitory effect was only observed in experiments with minced irradiated rootlets, the dose dependence of the effect was similar to that with joint cultivation of the irradiated and unirradiated seedlings. The results of the determination of the degree of staining of the medium by bromothymol blue for the unirradiated rootlets and those irradiated with various doses are given in Fig. 2. The changes in the redox

properties of the medium increased with time and were maximal after irradiation of seedlings with a dose of 150 r.

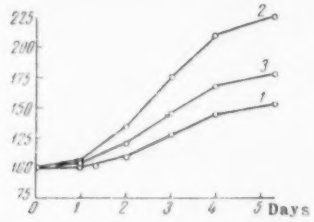


FIG. 2. Changes in intensity of staining of water by bromothymol blue indicator for irradiated and unirradiated bean seedlings. Abscissa, time in days; ordinates, intensity of staining. *1*, unirradiated seedlings; *2*, 150 r; *3*, 350 r.

In can be concluded from our results that inhibition of growth of unirradiated seedlings in joint cultivation with irradiated ones is associated with the vital activity of the latter. In summary; (A) — an effect was observed only on fairly prolonged joint cultivation (5–6 days) of both types of seedling, (B) — no effect was observed when the vital activity of the seedlings was strongly suppressed or ceased as a result of irradiation with high doses, (C) — no effect was present on destruction of the cells of the irradiated seedlings by grinding with sand.

Apparently, the factor inhibiting the growth of the unirradiated seedlings is not the direct consequence of irradiation but arises in the irradiated objects as a result of disturbed metabolism. This conclusion is confirmed by certain data in the literature. Thus, for example, the toxic properties of irradiated yeasts can be detected only after incubation in nutrient medium [4]. It is possible that the absence of an inhibitory effect exerted by the irradiated plants on the unirradiated ones described in the papers of certain workers [2, 3] may be attributed to the strong suppression of the vital activity of the plants by the excessively high doses employed, or their use of methods involving the destruction of the tissues of the irradiated plants.

The experiments with bromothymol blue show that the inhibitory influence of irradiated seedlings on unirradiated ones may be associated with change in the redox properties of the medium in which these seedlings are grown. The maximal changes in staining of the medium by the indicator are observed precisely at those times and for those doses of radiation at which appreciable inhibition of the growth of the detector seedlings occurs. For the same times and the same doses application of the potentiometric method has revealed in the water from the irradiated seedlings the greatest alkalization and reduction in the redox potential [5].

## SUMMARY

(1) The detected inhibition of unirradiated by irradiated seedlings is characterized by the presence of an optimum dose.

(2) The factor of inhibition apparently is not a direct product of irradiation but appears as a result of disturbances in the metabolism of the irradiated plants.

(3) The inhibitory influence of irradiated seedlings on unirradiated ones bears a relation to the changes in pH and the redox potential of the medium in which the seedlings are grown.

*Translated by* A. CROZY

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VOL.
5
1960

# REACTION OF THE PLANT TO INJURY AFTER X-IRRADIATION*

I. M. VASIL'EV and B. G. ZHUKOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

(*Received* 10 *April* 1959)

IF a platinum electrode be inserted into the tissue of a healthy vitally-active plant and the redox potential (r.p.) thereupon measured, it will be negative or close to zero. But already within 2–3 min the r.p. becomes higher and in the ensuing 30–40 min will continue to rise. The low initial r.p. reflects to a certain extent the intensive reduction processes occurring in living, vitally-active cells. The subsequent rise in r.p. is an indication of the response of the plant to injury. Morphologically, this response is expressed in the formation on the surface of the wound of a small encrustation insulating the damaged cells from the surrounding medium and which with time is transformed into a plug. This process is always connected with intensified oxidation [1, 2].

In our investigation we were concerned with the problem of how X-irradiation affects this response of plants to injury. The best criterion of this, in our view, is electrometric determination of the r.p.

## METHODS

The polymethyl methacrylate chamber depicted in Fig. 1 was used for the investigation. Three platinum electrodes were mounted in the chamber, which also contained

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a calomel electrode and a Petri dish with Knopf's nutrient solution to which were added the plants. The calomel electrode was connected to the nutrient solution by an agar-agar bridge. The platinum electrodes were inserted into the stems of the plants

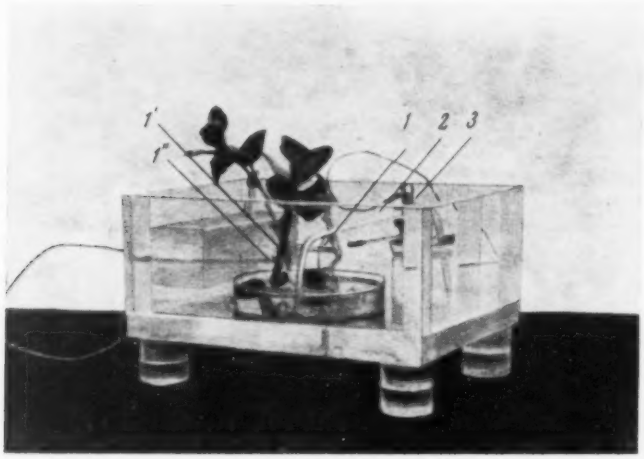


Fig. 1. General view of apparatus for determination redox potential in plants *1*, *1'*, *1''*, platinum electrodes mounted in polymethyl methacrylate box; *2*, agar-agar bridge; *3*, calomel electrode. At the bottom of the methyl methacrylate box there is a Petri dish containing Knopf's nutrient solution in which are placed the plant roots. In the dish three broad bean plants are seen with electrodes inserted.

and securely fixed. The circuit was closed by a PL-5 potentiometer. The plants were irradiated with a RUM-3 X-ray unit run at 165 kV and 15 mA, without a filter. Broad beans and haricot beans were used for the experiments.

## RESULTS

In Fig. 2 are given the results of the initial experiments with determination of the Eh for dead plants, killed by ether, and for living, 2-week old, plants. Several successive determinations of Eh were made after inserting the electrode into the stem. The first determination was made immediately after insertion of the electrode, the others at 2–3 min intervals.

As can be seen, the Eh for the killed plants was constant and did not vary with time in successive determinations. The picture for living plants was different. For the broad beans immediately after insertion of the electrode, the r.p. was negative, it then quickly rose, became positive and kept on rising throughout the entire experiment, which lasted for about 40 min. For the haricot bean the r.p. was from the very outset always slightly higher than for the broad bean. But it increased in a similar manner to the broad bean and reached about 140 mV within 30–40 min.

Later we found that if the electrode was inserted into a different part of the stem after the first series of Eh determinations the initial Eh value in the first determination

after insertion of the electrode was greater, although the character of the subsequent changes was retained. If a third puncture was made and a replicate set of Eh determinations made for this site, then the initial Eh was still greater but with no change in the character of its subsequent course. The picture described was repeated whatever

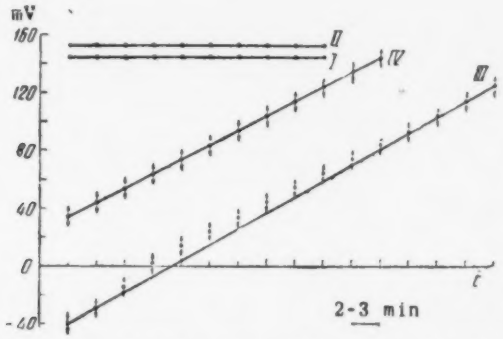


FIG. 2. Changes in redox potential. *I*, in broad bean plants killed by ether; *II*, in haricot beans killed by ether; *III*, in living broad bean plants; *IV*, in living haricot beans.

the site of the puncture in the stem. Fig. 3, in which the results of successive Eh determinations are given for broad beans and haricot beans illustrates this point. In some cases the determinations of the Eh and consequently the punctures were made in the order: base – centre – upper part of the stem; in others: centre – base – upper part of the stem.

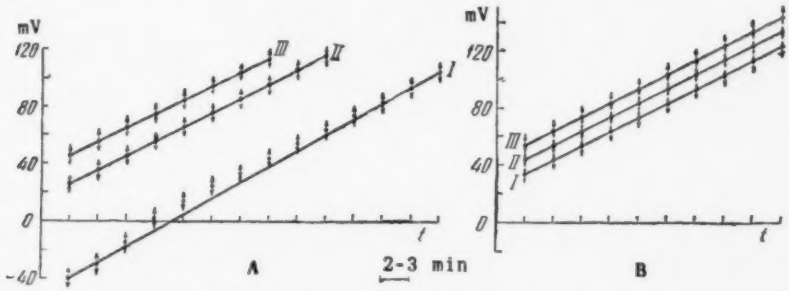


FIG. 3. Change, in redox potential on successive injury of plants. *I*, first puncture; *II*, second puncture; *III*, third puncture. Each curve represents the results of several determinations of the r.p. for different sites on the stem. *A*, broad beans, *B*, haricot beans.

The experiments described established that the living plant unlike the dead one reacts to wounding by a rapid rise in the r.p. It was found that the initial value of the r.p. varied for broad beans and haricot beans, being lower for the former. After each subsequent wound this value became increasingly higher and was particularly conspicuous for broad beans and less so for the haricot beans.

We then turned our attention to the main problem: how plants react to wounding after X-irradiation. In Fig. 4 the results are given of Eh determinations for broad beans made immediately after irradiation with a dose of 5000 r. There were no appreciable differences in the initial values for the Eh nor in its subsequent variations in the irradiated plant as compared with unirradiated plants. Irradiation with a dose of 5000 r did not affect the plant's response to injury.

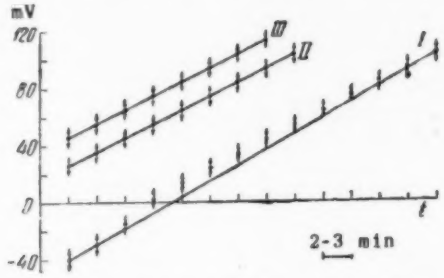


FIG. 4. Change in redox potential in broad beans immediately after irradiation with dose of 5000 r. *I*, first puncture; *II*, second puncture; *III*, third puncture.

A different picture was observed when the r.p. was determined several days after irradiation of the plants when the plastic substances not expended in growth had accumulated in the plants [3–6]. Fig. 5 gives the results of Eh determinations in the stem of broad beans 4 days after irradiation of the plants with doses of 5000 and 200,000 r.

Compared with the initial values for the Eh obtained immediately after insertion of the electrode into the stem in the experiments described above, the Eh values in this case were considerably higher. The r.p. was never negative. The rise in the potential during successive determinations of Eh for the same injury started at a higher level.

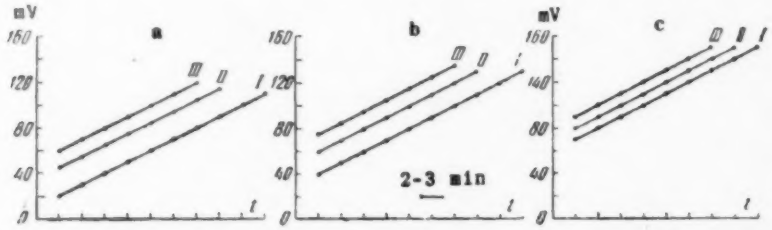


FIG. 5. Change in redox potential in broad beans 4 days after irradiation. *a*, plants irradiated with dose of 5000 r; *b*, plants irradiated with dose of 200,000 r; *c*, depressed plants irradiated with dose of 200,000 r. In all cases *I*, first puncture; *II*, second puncture; *III*, third puncture.

Another notable feature was the absence of any significant differences in the response to injury of the stems of plants irradiated with a dose of 5000 r and 200,000 r. In this case also the plants did not differ in external appearance. When for irradiation a

somewhat depressed plant was used, the initial Eh value after insertion of the electrode was higher (Fig. 5 (c)).

Eh determinations were repeated for the plants with an increased time lapse after irradiation. Both broad beans and haricot beans were investigated and irradiated with a dose of 100,000 r. The Eh values were determined 7 days after irradiation. As can be seen (Fig. 6), the changes in the r.p. for the irradiated plants as compared with unirradiated ones was repeated. Both for the broad beans and the haricot beans irradiated with a dose of 100,000 r, the initial Eh values determined on insertion of the electrode into the stem 7 days after irradiation and also the subsequent values were higher.

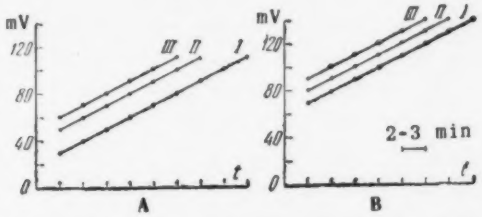


FIG. 6. Change in redox potential in plants 7 days after irradiation with dose of 100,000 r. *I*, first puncture; *II*, second puncture; *III*, third puncture; *A*, broad beans; *B*, haricots.

## DISCUSSION OF RESULTS

The experiments outlined above show that injury to plants by insertion of a platinum electrode into the stem causes the plant to respond with an increase in the initial r.p. The initial value of this potential bears a relation to the nature of the plant and to its physiological state. In broad beans it was always less than for haricot beans, and in the unirradiated plants it was negative. Later the r.p. rose rapidly. After infliction of each succeeding injury the initial potential was higher. A depressed state of the plant also raised the initial potential.

Immediately after irradiation of plants no appreciable changes in the initial value of the potential nor in its subsequent rise were observed. But a few days after irradiation the changes were clearly manifest. They were expressed in the fact that the initial Eh value after injury was higher. This effect was much the same both after irradiation with a dose of 5000 r and also after doses of 100,000 and 200,000 r.

We shall now discuss the change in r.p. observed in our earlier investigations with X-irradiation of potato seedlings [7] and now confirmed for broad beans and haricots. Electrodes "implanted" into the tissue of the seedlings were used and were inserted one day before irradiation of the plants in order to exclude the response of the plants to injury as described in this article. In these conditions the r.p. rose sharply from the start of irradiation but then as irradiation continued dropped to normal. Soon after irradiation ceased no significant changes could be observed in the r.p. for the irradiated as compared with the unirradiated plants.

This, then was the outcome of the experiments to determine the r.p. in irradiated and unirradiated plants taken as an indicator of the response of the plants to injury. It follows from this that immediately after irradiation the reaction of the plants to injury remains unchanged. But a few days after irradiation a change is seen in that the initial r.p. is higher.

Let us compare this result of the action of X-irradiation on plants with earlier results. It has been established that irradiation in itself even at very high doses of the order of many tens and hundreds of thousands of roentgens does not materially affect the permeability of the protoplasm, mineral uptake nor photosynthesis [8, 9]. Only growth is disturbed. At a dose of about 3000 r growth is completely suppressed as can readily be demonstrated by anatomical analysis of the growth points; there is no new formation of cells at this and higher doses [10]. Suppression of growth leads to accumulation of the products of photosynthesis and the minerals not utilized for growth [3, 5, 6, 9]. This is the precise cause of the gradual lowering of physiological activity of the plants and their death. The reaction of plants to injury gives additional confirmation to this point of view. Immediately after irradiation, as we have seen, no changes in this response were detected. The changes occurred only after several days had elapsed following irradiation when the plastic substances not used for growth had accumulated and the changes were expressed in a rise in the initial r.p. This means that the reduction processes are diminished and this is true for all cases of diminished vital activity of cells, including cases in which the cells are overfilled with "ballast" from the plastic substances hindering their normal life.

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VOL
5
196

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# STUDY OF THE ELECTRICAL CONDUCTIVITY OF THE LIVER OF IRRADIATED ANIMALS*

YE. V. BURLAKOVA and O. R. KOL'S

Faculty of Fundamental Biology of the Lomonosov State University, Moscow

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THE investigation of elementary physico-chemical processes developing in the tissues of irradiated animals is of great interest in that it can help to explain the mechanism of damage to live objects by ionizing radiation. The literature contains a number of papers devoted to the study of the primary physico-chemical processes of radiation injury [1,2]. The method of electrical conductivity is one of the various methods applied to investigate the physico-chemical changes produced by irradiation in animal tissues. This method is widely used in scientific research to study processes in living cells and tissues occurring as the result of changes in the physiological state due to the application of a number of chemical substances and physical factors and in the evaluation of a number of pathological processes [3].

The electrical conductivity method has also been used in the assessment of processes induced by X-rays in the tissues. Thus, it has been used in the study of changes occurring in the surviving tissue of the liver of guinea-pigs irradiated with lethal doses of X-rays [4]. This work demonstrated a decrease in the general resistance — "impedance" of the guinea-pig liver after irradiation.

The ratio $\rho/\omega$ for cancerous and normal tissues of X-irradiated mice has been investigated and the polarization properties of the tissues of the irradiated animals shown to be diminished [5]. Change in the electrical properties of the skin were established on X-irradiation of animals, namely a fall in the polarization, ohmic resistance and capacitance [6, 7]. A study was made [7], in the latent period of radiation sickness, of the electrical conductivity of the subcutaneous tissue of guinea-pigs and white mice irradiated with lethal doses of X and $\gamma$-rays. In most of the experimental animals the ohmic resistance was reduced by 20–30 per cent, the capacitance measured in the region of audio frequencies was increased and that measured at higher frequencies reduced; there was also a reduction in the tangent of the loss angle. An investigation of the electrical conductivity of the liver and spleen homogenates of white rats irradiated with lethal doses of $^{60}Co$ showed an increased electrical conductivity and the disappearance of impedance dispersion [8]. Investigation of the impedance changes of the rabbit brain on irradiation with X-rays with doses of 100–5000 r showed that ionic shifts could be observed in the brain even with very small doses of radiation, i.e.

* Biofizika **5**: No. 2, 194–201, 1960.

100 r [9]. In this work a fall in impedance of 1–2 per cent was noted in the early stages of irradiation followed by a 3–7 per cent increase. The author distinguished four phases of change in the electrical resistance and capacitance of the rabbit brain both on irradiation of the head of the animal and of the abdominal region. Some differences have been noted in these changes in relation to dose, dose rate and the localization of application. From the results of experimental work some of these authors have concluded that the method of electrical conductivity allows certain inferences to be drawn on the changes in the physico-chemical processes in living tissues on irradiation. Thus in the earlier papers [4–6] all the changes in the polarization properties of the tissues of the irradiated animals were ascribed in the main to changes in the permeability of semi-permeable membranes.

In later work, the changes in the electrical parameters of the tissues of irradiated animals were interpreted from other standpoints. Polivoda, for example, on the basis of investigations of the electrical parameters of the cell on irradiation considered that with the development of radiation injury in the latent period changes in molecular structure occur which are connected with disintegration and depolymerization of integral parts of the structure. The result is an increased number of ions and structures of low molecular weight [7]. The absence of dispersion in electrical conductivity found by Polivoda and the appearance of an anomalous variation of conductance with concentration of the liver and spleen homogenates of the irradiated animals, in the view of this author, indicated a decrease in the dipole moment of certain molecular structures [8]. Aladzhalova [9] considered that the impedance change of the rabbit brain on X-irradiation was suggestive of initial ionic shifts in the cortex due to the action of radiant energy on the receptors, the subsequent changes in the electrical conductivity in the rabbit cortex being the result of the disturbed metabolic processes. However, despite the above-mentioned studies on changes in resistance and capacitance of tissues of irradiated animals, the problem of the change in the electrical parameters of living tissues on irradiation is still not finally resolved and calls for further research.

The present work is devoted to a study of the influence of ionizing radiation on the high frequency and low frequency resistance and capacitance and the dispersion of resistance of the liver of irradiated animals and the change in these parameters on prolonged survival of the tissue.

## METHODS

The work was carried out on the liver of white mice irradiated with γ-rays from radioactive cobalt. The mice were irradiated with a GUBE-800 unit. The dose of radiation was from 800 to 5000 r at a dose rate of 600 r/min. Most of the experiments (90 per cent) were on mice irradiated with 800–1000 r at a dose rate of 600 r/min.

We used 260 mice, half of which served as controls. The electrical conductvity and capacitance were measured on a special apparatus designed to determine the resistance of living objects. The apparatus was designed according to the principle of bridge circuitry with a tuned output which enabled us to measure the ohmic and capacitance component of the total resistance (impedance) of the object on the basis

of an equivalent substitution circuit with parallel inclusion of resistance and capacitance. The bridge was supplied by a *R.F.* generator with a wide frequency range and zero phase difference. The generator gave a sinusoidal current the amplitude of which was stabilized by negative feedback. An oscillograph was used as the null instrument of the bridge; the standards of the known arm were resistance and capacitor boxes. The supply source of the generator was a well-stabilized rectifier. The resistance and capacitance of the living tissue was measured over a wide frequency range — from 30 kc/s to 1 Mc/s. The absolute error of measurement was 1 per cent. The variation in resistance with frequency was measured at the frequencies: 35, 50, 120, 200, 400, 600, 800 and 1000 kc/s.

The animals after irradiation were kept in the animal house in the same conditions as the controls. For the experiment the control and irradiated mice were killed by decapitation, exanguinated, the liver removed, washed with saline, slightly dried with filter paper and placed on tangential platinum electrodes in a humid chamber. The preparation of the livers of the test and control animals was carried out simultaneously and in completely identical conditions. In most experiments the large lobes of the liver were taken for the resistance measurements.

The first measurements of the resistance and capacitance were made 15 min after decapitation of the animals, these values being taken as the base line. Subsequent measurements were made after 30 min, 1, 2, 3 and 4 hr.

## RESULTS

The experiments yielded a well-defined pattern of change in the values of the high frequency and low frequency resistance and capacitance of the liver on irradiation of the mice with $\gamma$-rays from radioactive cobalt ($^{60}Co$). Thus, in all the experimental series we found a decrease in the low frequency resistance and capacitance and increased resistance measured at high frequencies. These changes started in the first few days, in some experiments within the first hours after irradiation and persisted to the day of the animal's death. On the first day after irradiation in 66 per cent of the experiments there was a decrease in the low frequency resistance and capacitance and in 80 per cent an increase in the high frequency resistance.

In the first days after irradiation changes in the electrical parameters were comparatively minor. Thus, in calculating the mean values for all the experiments of all the series (including the measurements on the days of least change) the low frequency resistance was found to be reduced by 7 per cent, capacitance by 10 per cent and with an 8 per cent increase in the high frequency resistance. On the days of the death of the animals these changes, as a rule were much greater. For example, in experiment 4 of the 6th series these changes on the day of death of most of the irradiated animals were expressed in the following values: low frequency resistance reduced by 18 per cent, capacitance by 32 per cent and high frequency resistance up by 14 per cent. In experiment 6 of the 7th series the low frequency resistance fell by 28 per cent, capacitance by 35 per cent with a 32 per cent increase in the high frequency resistance.

It may be concluded from the results of the experiments that ionizing radiations produce changes in the electrical parameters of the liver tissue, i.e. change in polarization properties. The best indication of the polarization properties of living tissues is the size of the polarization coefficient widely used in biological literature, a value which expresses the ratio of the resistance of the tissue measured at low frequency ($R_{l.\,f.}$) to the resistance at high frequency ($R_{h.\,f.}$) [10]. The application of the polarization coefficient to the study of the polarization properties of living tissues has a certain advantage since errors in the measurement of the absolute values of resistance, which might result from the differences in the areas of contact of tissue with the electrodes, are concelled out.

Therefore, to confirm our conclusions on the changes in the polarization properties of the tissue of the irradiated animal we calculated the polarization coefficient of the liver of the control and test animals. It was computed from the formula $K = \frac{R_{l.\,f.}}{R_{h.\,f.}}$ (in our work 35 kc/s and 1 Mc/s). As was to be expected from the experimental measurements of the values of the low frequency and high frequency resistance, the polarization coefficient fell as radiation sickness developed. This fall was slight in the first days and increased with the development of the disease. We calculated both the mean polarization coefficient for each series and the mean coefficient for five series. Thus, the mean polarization coefficients for the 1st, 2nd, 3rd, 4th and 5th series were: 2·4, 2·85, 3·0, 2·7 and 3·1 and for the controls respectively, 3·35, 3·8, 3·0, 3·15 and 3·7. The mean for the coefficients of all series for the controls was 3·3 and for the test, i.e. for the liver of the irradiated animals, 2·7. Thus, the polarization coefficient of the liver of the irradiated animals was less than that for the normal liver; this confirms the decrease in the polarization properties of the living tissues on exposure to ionising radiation.

Reduction in the polarization properties of living tissues on irradiation is also indicated by the experiments in which we determined the variation of the resistance of the liver with the frequency of current. In studying this aspect sometimes the resistance is expressed, in the literature, in reciprocal ohms, i.e. a relation between the value of the electrical conductivity and the frequency is established and this relation is termed the dispersion of the electrical conductivity of living tissue. In this work we also use the term dispersion, but by this we do not mean the value of the resistance in unit of electrical conductivity, but in ohms. Therefore, in this paper we speak of dispersion of resistance. In our experiments, the decrease in dispersion was already noted within the first hours of irradiation. The least dispersion in resistance was observed on the days on which the animals died. Fig. 1 shows the change in the dispersion of resistance of the experimental and control liver (experiment 8 of the 6th series). The abscissa is the logarithms of the frequency (in kc/s), the ordinates, the resistance in ohms. The unbroken curves denote the resistance of the liver in the controls, the broken ones the tests. Curves 1 and 2 denote the resistance of the liver 15 min after decapitation of the animals, curves 3 and 4, 5 and 6, and 7 and 8 after 1, 2 and 3 hr. As curves 1 and 2 show, the initial steepness in the dispersion for the experimental

liver is less than that for the control liver, which indicates a reduction in the polarization properties of the liver tissue of the irradiated animals. Thus, our conclusion that there is a reduction in the polarization properties of the tissues on irradiation of animals with γ-rays drawn from measurements of the absolute value of the high frequency and low frequency resistance and capacitance is also confirmed by determination of the polarization coefficient and the dispersion of resistance.

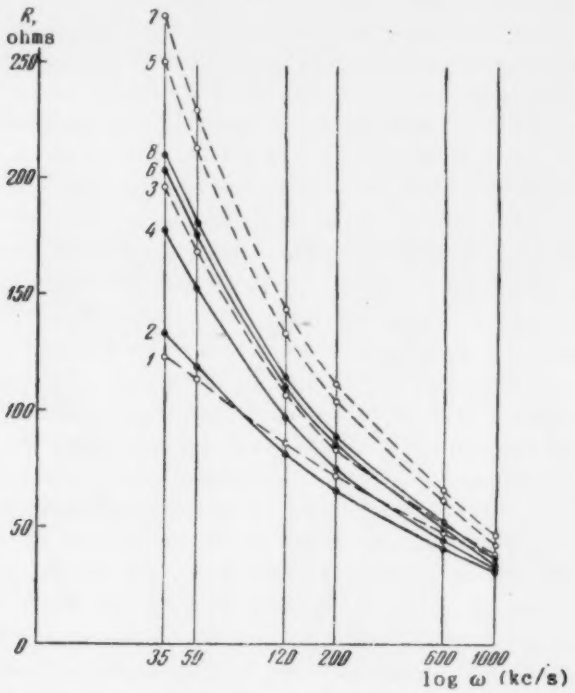


FIG. 1. Change in dispersion of resistance of liver on survival of tissue. Curves *1*, *3*, *5*, and *7*, resistance of liver 15 min, *1*, *2*, and 3 hr after decapitation of the animal; curves *2*, *4*, *6* and *8* same for controls.

There are grounds for assuming, from the changes in the electrical parameters of the tissues obtained by us, that on irradiation processes develop in the tissues involving an increased amount of low molecular weight substances. We estimated this increase in the amount of low molecular weight substances in the tissues of the irradiated animals from the changes in the values of resistance at low frequency and the polarization. This assumption is in agreement with the conclusions in one of the papers quoted [7] in which the author investigated the change in the low frequency resistance, capacitance and tangent of the loss angle for the subcutaneous tissue of irradiated guinea-pigs and mice. However, the results of our work do not confirm the conclusions drawn in paper [7] with regard to an increase in the number of free ions in the tissues on irradiation of animals. The increase in the high frequency resis-

tance of the liver established by us indicates a decrease in the number of free ions. In view of the conflicting findings on this point it was of interest to make a more careful study of the changes in the high frequency resistance of various tissues of irradiated animals over a wider frequency range.

In the literature there have for some time now been indications that with prolonged post mortem measurements made on various tissues of different animals [11, 12] the low frequency resistance is increased in the first hours followed by a drop on the 2nd and 3rd days. This increase has been interpreted by the authors of the papers mentioned merely as a post mortem decrease in the permeability of the semi-permeable membranes; it has still not been more precisely explained. Furthermore, there is no information at all on the change in the high frequency resistance and dispersion of resistance of surviving tissues, which might help to explain the essence of the physico-chemical processes responsible for the rise in the polarization properties of the tissues. Accordingly, we made a detailed investigation of the change in the values of the low frequency and high frequency resistance and capacitance and the resistance dispersion of the surviving liver tissue of normal animals and animals irradiated with radioactive cobalt. Differences in the change in these values with time were found for normal liver and for the liver of the irradiated animals in all the experiments.

The resistance dispersion of the surviving liver varied with time (Fig. 1). The absolute values of the resistance both of the test and control livers measured over a wide frequency range increased with time and the entire dispersion curve was moved upwards; this increase in the values of the resistance was most vividly marked in the 1st and 2nd hr and then during the 3rd hr slowed down. The greatest changes occurred in the region of low frequencies. The resistance dispersion of the liver of the irradiated animals increased with the time of its survival to a much greater degree than for the control liver. In other words, the processes in the surviving liver of irradiated animals leading to changes in the polarization properties of the tissues are completed to a higher level and at a higher rate than in the surviving liver of normal animals.

Fig. 2 depicts the curves of change in the resistance and capacitance measured at the frequency of 35 kc/s and resistance measured at a frequency of 1 Mc/s for the surviving liver of the test and control animals. The abscissa gives the time and the ordinates the mean values of the resistance (ohm) and capacitance ($\mu\mu$F) of all the measurements in the experiments of the 2nd series. The unbroken curve denotes the liver of the controls, the broken the test animals. These curves show that on survival of the liver there is an increase with time in the low frequency and high frequency resistance with a reduction in capacitance. In the experimental animals the resistance showed a greater and more rapid increase. For convenience Fig. 3 shows the change in the low frequency and high frequency resistance with time in percentages of the initial resistance for the same experimental series as in Fig. 2. The abscissa gives the time, the ordinates the percentage rise in resistance. As the initial resistance we took the first measurements of the resistance made 15 min after decapitation of the animal.

These graphs give a clear idea of the difference in the change in resistance with time for the control liver and for the liver of the irradiated animals and confirm the

results of measurements of the dispersion of resistance of the surviving liver tissue. This shows a comparatively minor difference in change of the resistance at high frequency and a sharp difference for the change in low frequency resistance. The low frequency resistance in the test animals increased at a faster rate and reached larger values. For the purpose of analysis and objective appraisal of the rate of the processes of change in the low frequency resistance for the controls and test animals the results were treated mathematically. The rate of the process is characterized by the first derivative of the resistance with time $v = \frac{dR_{l.f.}}{dt}$.

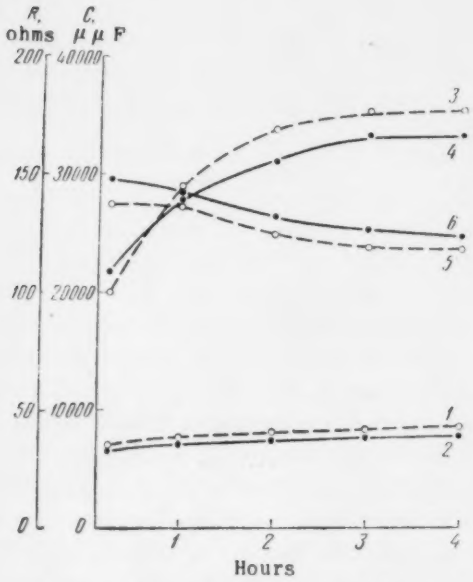


FIG. 2. Change in high frequency and low frequency resistance and capacitance of liver at different times after the death of the animal. Curve *1*, resistance at frequency of 1 Mc/s, test; curve *2*, same for controls; curve *3*, resistance at frequency of 35 kc/s, test; curve *4*, same for controls; curve *5*, capacitance at 35 kc/s, test; curve *6*, same for controls.

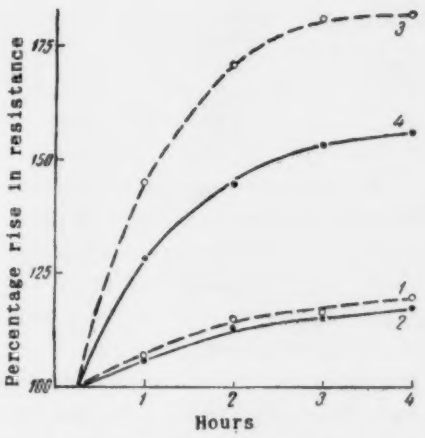


FIG. 3. Rise (as percentage of original value) of high frequency and low frequency resistance of liver. Curve *1*, rise in resistance at frequency of 1 Mc/s, test; curve *2*, same for controls; curve *3*, rise in resistance at frequency of 35 kc/s, test; curve *4*, same for controls.

The table lists the derivatives $v_1$, $v_2$, $v_3$, $v_4$ and $v_5$, calculated for change in the value of the low frequency resistance in time from the mean values for the 5th, 6th, 7th and 8th series and experiment 8 of the 6th series.

The value of the derivative $v_1$ expresses the rate of change in the low frequency resistance after 22 min; $v_2$ after 45 min, $v_3$ after 75 min, $v_4$ after 105 min and $v_5$ after 150 min. It follows from the values in the table that the rate of change in the resistance in the tests was greater than for the controls. This difference in rate was, in the main, observed for up to 2 hr, then the change in the value of the low frequency resistance

of the controls and tests proceeded at the same rate. An increase in the difference in the rates of change of resistance of the controls and test was observed on the days on which radiation injury developed; this difference was greatest on the day of the death of the animals. Therefore, difference in the derivates of the controls and tests computed for the mean value of the resistance of all experiments of a series will be less than that of the derivatives for the controls and tests computed for the experiments with the liver taken from the animal with radiation sickness already developed (experiment 8 of the 6th series, $v_5$ in the table).

TABLE 1

| $v_1$ | Average for 5th series | | Average for 6th series | | Average for 7th series | | Average for 8th series | | Experiment 8, 6th series | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Test | Control | Test | Control | Test | Control | Test | Control | Test | Control |
| $v_1$ | 1·8 | 1·8 | 1·47 | 0·4 | 1·2 | 1·1 | 1·7 | 1·4 | 2·6 | 1·2 |
| $v_2$ | 1·8 | 1·0 | 1·49 | 0·8 | 0·9 | 0·7 | 0·9 | 0·8 | 1·17 | 0·9 |
| $v_3$ | 1·0 | 0·9 | 1·3 | 0·9 | 0·7 | 0·5 | 0·5 | 0·7 | 1·33 | 0·7 |
| $v_4$ | 0·33 | 0·2 | 0·7 | 0·67 | 0·3 | 0·2 | 0·6 | 0·4 | 0·47 | 0·17 |
| $v_5$ | 1·05 | 0·05 | 0·21 | 0·2 | 0·02 | 0·02 | 0·3 | 0·1 | 0·33 | 0·1 |

As was stated above, on survival of tissues taken from normal and irradiated animals, the high frequency and low frequency resistances rise and the capacitance falls. Apparently, these processes run parallel to the processes of autolysis. Since the change in the electrical parameters of the surviving liver of the irradiated animals differs in magnitude and rate it may be assumed that the rate of autolytic processes in tissues of irradiated animals increases. Possibly this increase is the result of disintegration due to radiation of a number of substances which inhibit autolysis in the tissues of normal animals. To solve the problem as to what is the true link between the processes of autolysis and changes in the electrical parameters a special investigation of the parallelism of these processes is needed and is now, in fact, under way.

## SUMMARY

(1) The effect of γ-rays of radioactive cobalt on the value of the high frequency and low frequency resistance and capacitance and on the dispersion of resistance of the liver tissues of white mice has been studied.

(2) The action of lethal doses of γ-rays caused a decrease in the dispersion of resistance, the low frequency resistance and capacitance and an increase in the high frequency resistance of the liver of white mice as compared with the corresponding parameters in normal animals.

(3) The experimental results suggest that in the liver of irradiated animals there occurs an increase in the amount of low molecular weight substances and a decrease in the number of free ions.

(4) With long survival (3–4 hr) of the liver in the control and irradiated white mice there was a continuous change in the electrical parameters, namely increase in

the dispersion of resistance, the values for the high frequency and low frequency resistance and a reduction in capacitance.

(5) On prolonged survival of the liver of irradiated animals the change in the electrical parameters reached large absolute values and developed at a higher rate than for the surviving liver of the control animals; this seems to suggest intensified processes of autolysis in the tissue of irradiated animals.

*Translated by* A. CROZY

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# ROLE OF THE TIME FACTOR IN DAMAGE DUE TO RADIOACTIVE ISOTOPES*

IU. I. MOSKALEV

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IN the present communication an attempt is made to analyse the rôle of the dose rate and rate of application of radiation in the damage caused in certain reactions by radioactive isotopes introduced into the body.

A number of investigations undertaken with X and $\gamma$-irradiation of animals point to the existence of highly complex relations between the reactions of the body and the periods of application of the radiation. The basis for this lies in the different sensitivity of individual systems and organs to radiation, their different powers of compensation, recovery and summation of radiation damage and the functional interplay of organs and systems.

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Protraction of the dose usually leads to a decline in the effectiveness of radiation due to the presence of reparative processes which play an increased rôle as the dose rate is lowered.

The reparative processes come about in different ways in the different systems of the body so that reduction in the dose rate does not have the same importance for the different systems. Thus, for example, the ovaries have a high sensitivity to ionizing radiations and very weak reparative powers. In contrast to the haemopoistic organs and the mucous membrane of the intestines, the ovaries display uniform destructive lesions when exposed to either single massive or prolonged radiation [1]. The frequency of ovarian tumours changes little with dose rate or with its total value. It is increased both with daily irradiation of mice throughout their entire life with a dose of 0·11 r daily (total dose 90 r), and with a single application of 50 r [2].

The use of radioactive isotopes to study the rôle of the dose rate of radiation holds out much promise, and in some cases is more suitable than are external sources of radiation. It permits selective irradiation, for long periods of time, of the particular system or tissue within the organism after a single introduction of the isotope. However, in investigations concerned with the analysis of the biological effects of radioactive isotopes insufficient attention has been devoted to this problem. Yet the isolated communications where the results have been analysed from this standpoint show the important and sometimes decisive rôle of the time factor in the outcome of radiation sickness or a particular form of it.

Because of the lower dose rate and the inhomogeneity of irradiation due to the irregular distribution of the radiation source, single whole body X or $\gamma$-irradiation of rats (using $^{60}Co$ as source of the latter) is more effective than are incorporated radioactive isotopes. On irradiation with X or $^{60}Co$ $\gamma$-rays, the LD50/30 is 400–600 r, whereas from damage by radioactive isotopes it is 1·1–5·4 krep [3].

In the present work the criterion chosen for the biological effect of radioactive isotopes was the duration of life and the carcinogenic effect. An analysis is given of the results of experiments, undertaken jointly with V. N. Strel'tsova on the single administration to animals of different amounts of $^{90}Sr$ (859 rats), $^{144}Ce$ (641 rats) and also the results of other workers on the carcinogenic effect of $^{239}Pu$ [4–7]. The prerequisite of the investigations was to use different amounts of radioactive isotope in each experiment so obtaining different dose rates. Evaluation of the biological effect of the radioactive isotope in relation to dose rate was based on a comparison of the efficacy of uniform doses of radiation taken up by the tissue in different time intervals.

Table 1 presents information on the mortality of rats, as a function of total dose and the time of its deposition in the skeleton, from damage caused by chronically-effective amounts of $^{144}Ce$ and $^{90}Sr$. A perusal of this table shows that doses of radiation, up to 4·2 krad, taken up by the skeleton for any length of time (up to the 750 days) did not shorten the natural life of the animals. With a dose to the skeleton of 8·4–27·3 krad spread over 450–700 days more rapid death of the rats (on average by 15 per cent) was found. Accumulation of the same total dose of radiation but within a shorter time (50–350 days) made no difference to the life span of the animals. This

TABLE 1. MORTALITY OF RATS (IN %), IN RELATION TO THE TOTAL DOSE OF RADIATION (KRAD) AND THE RATE OF ACCUMULATION OF THE DOSE IN THE SKELETON, FROM DAMAGE CAUSED BY CHRONICALLY-EFFECTIVE AMOUNTS OF $^{144}$CERIUM AND $^{90}$STRONTIUM

| Time (days) during which relevant dose accumulated \ Dose (krad) | 0 | 0·5 | 0·8–1·0 | 1·7–2·5 | 4·2 | 8·4 | 16·8 | 27·3 | 51·0 |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $^{144}$Cerium | | | | | |
| 50 | 0 | 0 | 0 | — | — | 0 | — | — | — |
| 100 | 0 | — | 0 | 1·7 | — | 2·5 | — | 1·5 | — |
| 150 | 7 | 1 | — | 2 | 9 | — | — | — | — |
| 200 | 8 | 1 | — | — | — | — | — | 10 | — |
| 300 | 14 | — | — | — | — | — | — | — | 82 |
| 400 | 16 | — | — | — | 12 | 33 | — | — | — |
| 450 | 24 | — | — | — | — | — | — | — | — |
| 550 | 44 | 32 | — | — | — | — | — | — | — |
| 600 | 55 | — | — | 56 | — | — | — | 74 | 87 |
| 650 | 63 | — | 69 | — | — | — | — | — | — |
| | | | | $^{90}$Strontium | | | | | |
| 50 | 0 | — | 0 | 0 | 0 | 0 | 0 | — | — |
| 100 | 0 | — | — | 0 | 0 | — | — | — | — |
| 150 | 1·5 | — | — | 0 | 1·0 | — | 2·3 | — | — |
| 200 | 3 | — | 0 | — | 4·0 | 0 | — | — | — |
| 350 | 13 | — | — | — | — | 14 | — | — | — |
| 650 | 70 | — | 76 | — | 70 | 84 | — | — | — |
| 700 | 78 | — | — | — | — | — | 95 | — | — |
| 750 | 89 | — | — | 90 | — | — | — | — | — |

observation indicates that on application of low doses of radiation, protracted irradiation is more effective — if the life span is taken as the criterion of the biological effect and the effect is determined immediately after the accumulation of the given dose. This, at first sight, is contrary to the accepted view in radiobiology that protracted application is less effective than concentrated action. In considering the results it must be borne in mind that variations in the life span is one of the most complex reactions of the body to radiation with a high degree of compensation due to the presence of a number of powerful neuro-humoral compensatory mechanisms. Obviously, collapse and disturbance of these mechanisms at a given level of radiation does not appear immediately on cessation of the treatment but at later stages of the experiment against a background of physiological ageing which, as is known, is speeded up by irradiation [8]. It should also be remembered that death of the animal at any level of radiation is the result of the development of various pathological processes with a variable duration of the "latent" period, not taken into account in this case.

At higher levels of radiation, in contrast, intensive irradiation was found to be more effective. Thus, in the case of $^{144}$Ce on accumulation in the skeleton of a dose

of 51 krad over 300 days, 68 per cent more animals died than in the controls whereas accumulation of the same dose but over 600 days gave a difference in the mortality of only 22 per cent.

When exposed to small doses of radiation, the body makes use of various compensatory mechanisms as seen in the fact that on injury with low amounts of $^{144}Ce$ (1–5 mc per rat) a "stimulatory" effect appears which is manifest in the slower death of the animals as compared with good controls. With a total dose of 0·5–2·5 krad accumulated in the skeleton over 150–200 days after the introduction of $^{144}Ce$, the death rate of the animals in the experimental groups was 5–7 per cent lower than in the controls. With a total dose of 0·5 krad to the skeleton over 550 days, the difference in mortality was 12 per cent lower. These differences are statistically significant.

Slower death of the animals from damage due to $^{144}Ce$ was observed with dose rates of radiation in the skeleton of 0·8 and 3·3 rad daily on exposure for 550 and 150 days respectively; and in the case of 16·8 rad daily for 150 days. After uptake by the skeleton of a dose of radiation of 0·8–2·5 krad over a longer interval ($>$ 600 days) no stimulatory effect of radiation could be seen. The mortality of the animals in the experimental and control groups was the same and in individual cases even higher than in the controls. Lorenz, Heston, Eschenbrenner and Deringer [2] have also observed the slower death of mice as compared with suitable controls on daily irradiation with $^{60}Co$ $\gamma$-rays (0·11 r daily for 8 hr).

At later stages there were no differences in the times of death. These facts suggest that the "stimulatory" action of radiation is apparently of a temporary character and the destructive action accumulates with time even for low levels of radiation. The development of acquired immunity both to the normal microflora of the body and to the intrinsic denaturated proteins of the tissues [9, 10] is possibly one of the mechanisms responsible for the "stimulatory" action of radiation.

A characteristic feature of the biological effect of radioactive isotopes accumulated in considerable amounts in the skeleton is their ability to eventually cause tumours of bony and haemopoietic tissues.

We shall now consider the frequency of the appearance of bone tumours and leukaemias in rats, from damage by $^{90}Sr$ and $^{239}Pu$, in relation to the time of accumulation in the skeleton and bone marrow of suitable doses of radiation with a tumofacient action (Table 2).

Inspection of Table 2 shows convincingly that after damage by $^{90}Sr$ the frequency of osteosarcoma formation varies with the radiation dose rate. Within the limits of the same total doses (12·6–21·0 krad) a 20-fold reduction in the dose rate causes a 10-fold reduction in the frequency of osteosarcoma. Thus, on accumulation in the bony tissues of 12·6 krad over 30, 100, 400 and 650 days the frequency of osteosarcoma formation was 30, 8·4, 2·6 and 4·2 per cent respectively. At a radiation dose of 21·0 krad taken up by the skeleton over 50, 150 and 650 days osteosarcomas developed respectively in 34, 35 and 2·6 per cent of the rats. Within the limits of the optimal osteosarcomogenic doses (47·0 krad) a three-fold reduction in the dose rate of radiation had little effect on the overall frequency of osteosarcoma formation. The data

TABLE 2. FREQUENCY OF TUMOUR DEVELOPMENT IN RATS (IN %) AMONG ANIMALS SURVIVING 200 DAYS IN RELATION TO THE TOTAL DOSE OF RADIATION AND THE RATE OF ACCUMULATION, DAMAGE CAUSED BY $^{90}$STRONTIUM

| Effect | Radiation dose (krad) | Time (days) in which relevant dose accumulated | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 30 | 50 | 75 | 100 | 150 | 200 | 250 | 300 | 400 | 600 | 650 | 800 |
| Bone tumours | 12·6* | 30*** | — | — | 8·4 | — | — | — | — | 2·6 | — | 4·2 | — |
| | 16·8 | — | 32 | — | — | 25 | — | — | — | — | — | 2·6 | — |
| | 21·0 | — | — | 34 | — | — | 35 | — | — | — | — | — | 2·6 |
| | 47·0 | — | — | — | — | — | — | 35·8 | — | — | — | — | 47·0 |
| Leukaemia | 0·28** | — | — | — | — | — | 0 | — | — | — | — | 0 | — |
| | 0·55 | — | 0 | — | — | — | — | — | — | — | — | 3·3 | — |
| | 2·7 | — | 1 | — | — | — | 3·8 | — | — | — | 5·6 | — | — |
| | 5·6 | — | 2·0 | — | — | 4·5 | — | — | — | — | — | 8·3 | — |

N.B. * Dose of radiation to skeleton; ** dose of radiation to bone marrow; *** because the carinogenic effect is not immediately apparent but appears at the end of the "latent" period which on average is equal to 200 days, the tumour yield was determined not at the moment of accumulation of the given dose of radiation but at the end of this latent period. Thus, for a dose of 12·6 krad accumulated by the skeleton over 50 days, the frequency of osteosarcoma induced by this dose was determined on 250th day. It was arbitrarily accepted that prolonged irradiation of the tissue during the "latent" period did not affect the frequency of tumour development at this stage.

considered show that at low levels of radiation restorative processes occur in the structures responsible for the tumofacient degeneration of the bone cells with a resulting incomplete summation of the effect of radiation.

Unlike the case of osteosarcomas, prolongation of the time of delivery of leukaemia-effective doses of $^{90}$Sr raises the frequency of the onset of leukaemia (Table 2). It rose 4–6 times for a 12-fold reduction in the dose rate. Thus, for example, with a total dose of 2·7 krad to the bone marrow over 50 days, leukaemia developed in 1 per cent of the animals and in 3·8 and 5·6 per cent of the animals respectively on accumulation of this dose over 200 and 600 days. With a total dose of 5·6 krad the frequency of leukaemia development with accumulation over 50, 150 and 650 days was 2·0, 4·5 and 8·3 respectively. This observation is suggestive of summation and poor recovery of the damage in the cambial elements of the bone marrow leading ultimately to onset of leukaemia. The results of our observations are in agreement with those of Mole [11] and Kaplan and Brown [12] which indicated that on fractionation of the dose the leukaemogenic action of radiation is intensified.

It should be noted that the frequency of onset of leukaemia drops as the total dose of radiation is decreased. Thus, at a dose of 0·28 krad accumulated over 50 and 650 days, the frequency of leukaemia development was the same as for the controls and for doses of 0·55, 2·7 and 5·6 krad it was 3·3, 5·6 and 8·3 per cent respectively.

The different reaction of the bone and haemopoietic cells on prolonging the delivery of the carcinogenic doses of $^{90}$Sr is probably to be explained by the distinctive character of the irradiation of the cell. Unlike the bone cells, the cambial elements of the bone marrow are irradiated chiefly by electrons at the end of their range, pro-

ducing a much greater density of ionization than in the initial part of their track, and thus have a greater biological effectiveness. It might therefore be expected that damage from α-emitters, producing even greater ionization density, would increase the frequency of osteosarcoma formation with prolongation of the dose in a similar fashion to the leukaemia described above. Analysis of the experimental findings confirms this supposition (see figure). Unlike $^{90}Sr$ after damage by $^{239}Pu$ the frequency of osteosarcoma development increased as the dose rate was decreased. On accumulation in the bone tissue of a radiation dose of 0·8 krad over 200 days the frequency of osteosarcoma formation was 16 per cent; on accumulation over 650 days it was 30 per cent.

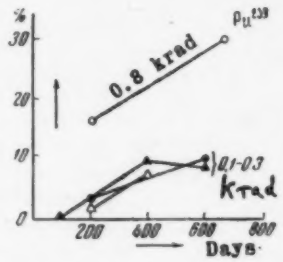


FIG. 1. Variation of frequency of onset of osteosarcomas in rats with dose rate and total dose of radiation in bone tissue ($^{239}Pu$).

The variation of the carcinogenic action of radiation with the type of radiation may also be illustrated from the frequency of osteosarcoma development in relation to the level of activity introduced (or dose taken up by the skeleton). The experimental data convincingly show that the range of osteosarcoma-producing doses from α-emitters is higher and the frequency falls considerably less with a decrease in dose than from β-emitters. Thus, according to the observations of Finkel in mouse experiments [6] with $^{226}Ra$, a 10-fold decrease in the amount of isotope introduced (from 60 to 6 μc/kg) led to a fall in the frequency of osteosarcoma formation from 60 to 35 per cent, i.e. it was halved; in the case of $^{239}Pu$ the frequency of osteosarcoma development fell by a factor of 4·6 on reducing the amount of the activity introduced ten times (from 3·0 to 0·3 μc/kg). The investigations of Bukhtoiarova and Lembyerg [4] with $^{239}Pu$ in rats showed that for a ten-fold decrease in the level of activity administered (from 6·3 to 0·63 μc/kg) the frequency of osteosarcoma development fell 2·5 times. Quite different relations are observed after damage by the β-emitters $^{90}Sr$ and $^{144}Ce$ [13]. In the case of these isotopes a ten-fold reduction of the optimal osteosarcomogenic doses (from 0·5–1·0 to 0·05–0·1 μc/g) led to a fall in the frequency of osteosarcoma development by 30–50 times, i.e. a greater reduction than in the case of α-emitters.

A noteworthy feature in connexion with the facts outlined is the observation of Kuzma and Zander [14] who found the soft β-emitter $^{45}Ca$ to be more effective than $^{89}Sr$ in inducing osteosarcomas on prolonged intake of these radioactive isotopes. The data examined show that the damage induced by α-particles is more poorly restored and is better summated with protraction of their action. The higher the ionization

density, the poorer the processes of recovery in the structures of the cells damaged by the mutagenic action of radiation. Therefore, α-emitters, soft β-emitters and also hard electrons at the end of their range cause damage which is not only summated in time, but in some cases is more prominent on protraction of the radiation. The possibilities of recovery and compensation vary for different cell formations and body systems. They depend not only on the rate of application of the radiation, but on the type of radiation. In the reaction of the body a major rôle is played by the time factor. Change in the dose rate leads not only to quantitative, but also to qualitative differences in the reactions.

## SUMMARY

(1) The biological action of radioactive isotopes depends on the dose, dose rate and the type of emission.

(2) On injury with $^{90}Sr$ and $^{144}Ce$ there exist doses with a temporary "stimulatory" effect (0·5–2·5 krad), others which do not affect the natural life span of the rats (up to 4·2 krad) and also others which shorten it. On prolonging the time of delivery of the doses of radiation of 8·4–27·3 krad, the effectiveness of $^{144}Ce$ as judged by the duration of life of rats is increased; on prolonging the delivery of higher doses (51 krad) it falls.

(3) After damage by $^{90}Sr$ the frequency of onset of osteosarcomas falls, and that of leukaemia increases, on lowering the dose rate. As distinct from $^{90}Sr$, with $^{239}Pu$ the frequency of osteosarcoma development increases on reducing the dose rate.

(4) As compared with β-emitters the damage induced by α-emitters is less readily restored and is better summated on prolongation of the action.

*Translated by* A. CROZY

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# MEASUREMENT OF ABSORBED DOSE IN AN INHOMOGENOUS FIELD OF RADIATION WITH AN EXTRAPOLATION CHAMBER WITH DIAPHRAGM*

G. B. RADZIYEVSKII

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
5
196

## INTRODUCTION

IN radiation work with comparatively weak sources of radiation the object to be irradiated must be placed in the direct vicinity of the source in order to obtain high dose rates. In this case, because of the inhomogeneity of the dose field, the objects are usually given the shape of thin flat films or layers. Accurate determination of the absorbed dose in such an object close to the source meets with serious difficulties because of the need for a dosimeter with a detector of small thickness. Application of the "difference" method [1, 2] which successfully circumvents these difficulties necessitates using a detector calibrated for complete energy absorption. Therefore, in practice it is not always convenient to use this method.

A good means of determining the absorbed dose in a thin flat layer close to the source is provided by the Failla extrapolation chamber [3, 4] with cylindrical measuring volume confined to the centre by the lines of force of the field. However, such a chamber, from the point of view of suitability for "near focus" measurements has certain defects. The rectilinearity of the extrapolation graph requires a uniform ionization density over the volume of the chamber, which in near focus measurements is approximately realized only if the distance between the electrodes is very small. In these conditions extrapolation methods cannot lay claim to any great accuracy. In addition, we can mention a case where in practice such an extrapolation method is impracticable. We have in mind measurement of soft (less than 60 kV) X-radiation close to the source. Despite the fact that the Bragg—Gray principle is applicable to this range of energies [5] it is in practice almost impossible to construct a "wall" operating system for the extrapolation chamber with variable clearance (distance between the electrodes) and with normal (not reduced) gas pressure because of the small values of the range of

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the electrons emitted from the walls. Chambers with higher clearances operated as a "gas action" systems are not possible because of the inhomogeneity of the radiation field close to the source.

In this work, to measure the absorbed dose in an inhomogenous field of radiation we used a flat ionization chamber with a variable volume and an unrestricted volume between the electrodes, employing the full cross-section of the effective beam of radiation emerging from the diaphragm. The idea of near focus measurements using the full cross-section of the beam was previously applied by Lea [6] for the special chamber he designed for soft X-radiation. The Lea chamber, intended for measurements of a definite type of radiation and in a definite position near the source, was not a thin cylindrical chamber and had the shape of a truncated cone; it was not fitted with a diaphragm and had a practically constant ionization volume. However, the principles underlying its construction are similar to those outlined in this paper.

VOL. 5 1960

## METHODS OF MEASUREMENTS AS APPLIED TO X-AND $\gamma$-RADIATION

The scheme of the ionization chamber is given in Fig. 1. The working volume of the chamber is practically unconfined by the sides. Only the beam of the primary radiation is limited (by the diaphragm). The diameter of the electrodes is such as to ensure practically complete utilization of the ionizing powers of the secondary electrons emitted within the chamber.

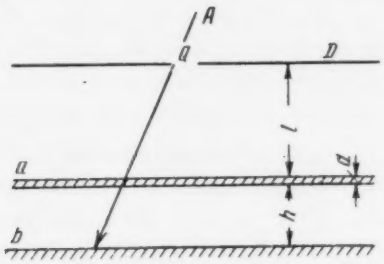


FIG. 1. Scheme of the ionizing chamber. *A*, extended radiation source; *D*, radiation screen with aperture of area *Q*; *a*, frontal wall of ionizing chamber of thickness *d* ($d > R_{max}$ where $R_{max}$ is the maximum range of secondary electrons); *b*, thick rear wall of chamber; *h*, depth of chamber, *l*, distance between diaphragm and frontal wall of chamber.

We shall first discuss the case of a homogenous chamber (i.e. with walls of air-equivalent material). We shall consider the thin horizontal (parallel to the plane of the diaphragm), laterally unconfined layers of material of the wall or the gas, located under the diaphragm. The layer thickness can be nominally expressed in mass per unit area of the layer. A unit layer is a layer the thickness of which corresponds to the unit of mass per unit area. If it is considered that the primary radiation is not attenuated or scattered in the wall, the intensity of the radiation absorbed in any unit layers of the walls and the intensity absorbed in any unit layers of the gas will equal each other.

The ratio of the ionic current $I$ in the chamber to the depth $h$ of the chamber remains in this case constant for any value of $h$. The value $I/h$ is a measure of the intensity absorbed per unit layer of material of either the wall or the gas, (homogenous chamber).

This follows from the geometrical conditions of absorption of each of the beams (narrow beams of radiation) emerging from source $A$. The geometry in this case is such that in each of the (unbounded) planes, parallel to the plane of the diaphragm, there will be the same flux of secondary electrons (by flux we intend the function giving the number and distribution in energies and directions of the secondary electrons intersecting the whole plane per unit time). In view of the equality of the fluxes of electron emission for all planes parallel to the diaphragm, the intensities absorbed in the unit layers will also be equal.

By finding from $I/h$ (or $dI/dh$) the absorbtion per unit layer, we can relate this value to the unit layer lying in the plane of the entry diaphragm $D$. Since for the unit layer lying in the plane of the diaphragm, we know the precise area on to which the beam of primary radiation impinges (this area is equal to $Q$), we are able to calculate the value of the absorbed dose in the material of the wall (in air). By dividing the amount absorbed per unit layer by the area $Q$ of the aperture of the diaphragm, we obtain the intensity of the absorbed dose, in the material of the wall ("air"), corresponding to the position of the object to be irradiated at the site of the entry diaphragm of the chamber. In other words, we obtain the intensity of the absorbed dose which would be received (in conditions of electron equilibrium) by a thin layer of the material of the wall (air) lying precisely at the position of the aperture of the entry diaphragm and irradiated by a wide beam in the absence of a diaphragm.

We shall now consider an inhomogenous chamber, i.e. with walls not equivalent to air. In such a chamber the proportionality between $I$ and $h$ will apply at sufficiently low values of $h$ when the chamber works as a "wall-action system". From the Bragg—Gray formula it is possible to arrive at the dose absorbed per unit layer of wall material from the dose absorbed per unit layer of gas between the electrodes, determined from $dI/dh$.

If we increase the height $h$, then for certain values of $h$ the "action of the wall" will gradually pass into the "action of gas". Starting with a sufficiently large value for $h$, the chamber will work as a "gas action" system. In this region, again, as with the "wall" system, $dI/dh$ is constant and from this value one can find the absorption per unit layer of gas. In view of the inhomogeneity of the chamber, the slope of the curve $I(h)$ in the wall region is not equal to that in the gas region. The curved portion of the graph corresponds to the intermediate values (between the wall-gas systems) of $h$. This transitional region is not of interest for dosimetry.

Working with an inhomogenous chamber we shall relate, as before, the values of the intensity absorbed per unit layer (material of wall or air) to the layer in the plane of the entry diaphragm and thus determine the corresponding intensities of absorbed dose.

VOL 5 196

So far we have not taken into account: (1) the attenuation of radiation between the entry diaphragm and the frontal wall of the chamber (we shall disregard the scatter along this path); (2) the absorption and scatter by the frontal wall of the chamber; and (3) the scatter by the rear wall.

We shall show how these factors are allowed for when using the chamber. A correction is introduced for attenuation of the radiation along the path between the diaphragm and the frontal wall (found experimentally or by calculation). As for the scatter of radiation in the walls of the chamber and absorption in the frontal walls, here two courses may be adopted. It is possible by one means or another to determine the influence of absorption and scatter on the intensity to be measured and to make the relevant corrections. However, one can take another course of action and select conditions of irradiation corresponding, as far as possible, to the conditions in which the measurements are made. This method may nominally be termed "modelling" of the conditions of measurement on irradiation. For the conditions of irradiation and measurement to agree, in our case it is necessary that the shape, size and position of the object in the field of radiation are exactly the same as those of the entry diaphragm of the chamber. Thereby, precisely that beam of radiation is measured which completely passes through the chamber and acts during irradiation on the object. Subsequently, the conditions of correspondence are satisfied if irradiation is carried out in a wide beam, through a filter which like the frontal wall of the chamber is located directly above the object and a (sufficiently thin) object is placed on a wide base made of a light atom material which scatters the radiation in the same way as the rear wall of the chamber. On irradiation in such conditions no corrections are made for absorption in the frontal wall of the chamber of for the scatter from both walls.

The radiation screen $D$ must be sufficiently thin, at least around the edges of the aperture $Q$, to allow one to disregard the marginal effects (scatter, fluorescence) of the diaphragm and to consider the action of the diaphragm as a purely geometric cut-out of the wide beam of radiation. This requirement limits the energy of radiation which can be measured by the method described and this limitation is greater the more divergent the beam of radiation, i.e. the closer to the source the measurement is made. The requirement for the full utilization of ionizing power of the secondary electrons also restricts the energy of radiation.

## DESIGN OF CHAMBER AND RESULTS OF MEASUREMENTS OF RADIATION FROM A BF-70 TUBE

The schematic diagram of the chamber is given in Fig. 2. The height of the chamber can be varied on rotation of bush 1 with a screw thread connected to the lower electrode 2 of the chamber. The height of the clearance $h$ in the chamber was set and measured by remote control, without switching off the apparatus from the control room of the X-ray unit, by means of a pair of selsyns. The accuracy of the variation of $h$ was about

0·01 mm. The zero reading of $h$ (zero clearance) was set by electrical contact between the electrodes of the chamber. The maximal value of $h$ was 20 mm. Both electrodes were made of polymethyl methacrylate covered with graphite. The thickness of the frontal wall, 7, was 1 mm. At the top the chamber was closed by lead casing, in the central aperture of which was placed the lead diaphragms. A section of the diaphragm is shown at the bottom of Fig. 2. The thickness of lead at the edges of the aperture of the diaphragm was $\sim 70\ \mu$. A set of diaphragms was used with apertures ranging from 3 to 25 mm in diameter. The proportionality between the ionization current in the chamber and the area of the aperture of the diaphragm was checked repeatedly. In all cases proportionality was observed with an accuracy of $\pm 1$ per cent (after correcting for the passage of radiation through the parts of the diaphragm adjacent to its aperture and having a thickness of 70 $\mu$ — correction up to 4 per cent).

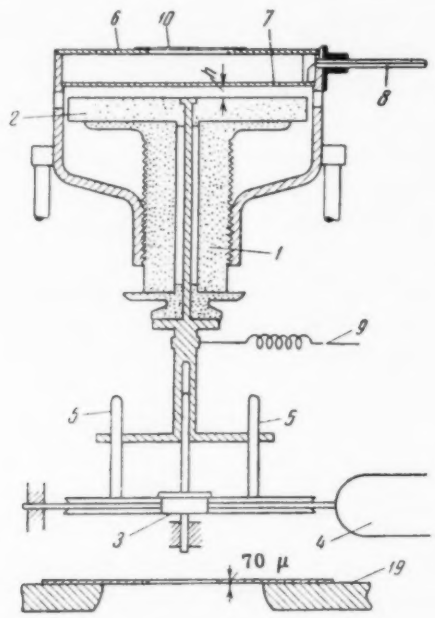


FIG. 2. Arrangement of extrapolation chamber with diaphragm. *1*, bush, screwed into frame of chamber moving upwards and downwards on rotation; *2*, lower wall of chamber; *3*, screw drive; *4*, selsyn rotating the screw drive; *5*, two fingers transmitting rotation to bush *1*; *6*, detachable lead screen with aperture; *7*, frontal wall of chamber; *8*, cable for leading off ionic current; *9*, contact head for transmission of voltage to lower electrode; *10*, lead diaphragm shown at bottom of figure in enlarged cross-section.

To measure the ionic current a galvanometer was used, the chamber potential being supplied by a screened cable. In all cases, the saturation conditions of the chamber were checked.

The measurements made by emission from a BF-70 tube (with beryllium window and with a ruled focal surface about 26 mm in length) consisted of two parts: (a) investigation of the mode of action of the chamber (to find the system of "gas action") and (b) the measurements proper of the dose field near the tube window. In all cases the tube was worked on the half-wave principle at 60 $kV_{max}$ with a current ranging from 0·1 to 40 mA.

*Investigation of the mode of action of the chamber*

The first measurements carried out with the graphite chamber (electron emission within the chamber was given only by the graphite layer on the electrodes) showed that as assumed, the graph of the variation of $I$ with $h$ has the form of a straight line. The height of the clearance was varied in these experiments from 1–2 mm to 20 mm and special measurements at very low clearance (0·2 mm–1 mm) were not made. Since in air the range of electrons with energy of 20–30 keV may reach 15 mm, it was not clear whether the straight line graph was attributable to the action of the "gas" or to the "walls" of the chamber.

To solve this problem two series of experiments were carried out. In one series the material of the walls of the chamber was changed while keeping constant the other conditions of measurement (voltage, current, geometry, filtration of radiation). Measurements were made with different materials for the walls of the chamber and with unfiltered radiation and radiation filtered with 0·5 mm Al. The following materials were used for the chamber walls; graphite (chamber of the usual design), aluminium (thin foil was fixed to the internal surfaces of the electrodes), copper (foil was fixed to the electrodes), water (a flat plate embedded slightly higher than the edges with 2% agar was placed on the lower electrode and a moist filter paper was attached to the internal surfaces of the upper electrode). We measured the function $I(h)$ for low values of $h$ (up to 1–2 mm).

One of the graphs is plotted in Fig. 3 giving the curve $I(h)$ for the Al chamber for unfiltered radiation. It is clear that for $h > 10$ mm the graph is linear, for $h < 10$ mm it is curved.

The results of this series of experiments may be summed up as follows: for each material of the wall and for each hardness of radiation (with filter or without), with the exception of the Cu chamber and filtered radiation (see text below and Fig. 5), the boundary value of the clearance ($h_r$) was found above which the graph $I(h)$ became linear. For $h < h_r$ the graphs are curved. In no case did we obtain linear graphs for low values of $h$, although $h$ was taken right down to 0·2–0·3 mm. For the chamber with "aqueous" walls the graph was straight over the entire range of variation in the height of clearance (0·4 mm $< h <$ 20 mm). An important factor was that the straight part of the graphs plotted for the different materials of the walls with uniform systems of operating the apparatus had uniform slopes. The results obtained may be explained only by assuming that for $h > h_r$ the chamber works as a "gas action" system and for 0·3 mm $< h < h_r$ as a transitional system. It was not possible to achieve a "wall"

system in any instance. As for the aqueous chamber the low values for $h$ corresponding to the transitional region were apparently never reached. In fact, for radiation from a tube with a Be window, working at 50–60 $kV_{max}$ a chamber with "aqueous" walls and with an air cavity is almost homogenous. We shall compare the spectra of radiation of the tube with the Be window for the two systems used (filtered and un-filtered) obtained by calculation from the results given in paper [7] with the graph for the ratio of the mass coefficients for true absorption in water and air (according to paper [8]). The comparison shows (Fig. 4) that doses absorbed in water and air (in conditions of electron equilibrium) cannot differ by more than 1–1·5 per cent for a given radiation. The sole cause of the inhomogeneity and the existence of a transitional region at the air–water boundary can in this case only be the difference in the stopping powers of water and air (difference ~ 12 per cent principally due to the hydrogen of the water). The thickness of this transitional region is apparently of the order of fractions of a millimeter in air and fractions of a micron in water.

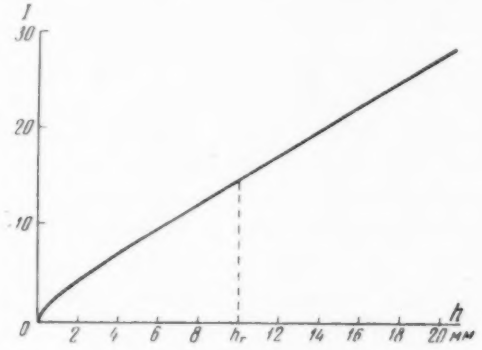


FIG. 3. Explanation in text.

To confirm the assumption that for $h > h_r$ the chamber works as a "gas action" system, a further series of experiments was carried out. In these experiments measurements with an extrapolation chamber with walls of different materials were compared with those of a normal chamber. All comparisons of the normal and extrapolation chambers were made with a diaphragm at a distance of 100–110 mm from the exit window of the tube. To reduce the recombination in the normal chamber, the measurements were made with an anode current of 0·1–1 mA. The ionic current from both chambers was measured, with an electrometer valve, from the drop in potential in a high ohmic resistance. The absolute magnitude of the resistance did not matter since the aim of the experiments was merely to compare the results of both chambers. At the field voltage in the normal chamber of 600 V/cm saturation was not in most cases reached. The saturation current was determined by voltage extrapolation [9, p. 27].

With the saturation current values obtained, corrections were made for: (a) the "transparency" of the parts of the diaphragm of thickness 70 μ adjacent to the aper-

ture (Fig. 2), the value of the correction (up to 4 per cent) was determined experimentally; (b) for attenuation of the beam of radiation in air, and in the normal chamber in the path between the entry diaphragm and the centre of the measuring electrode (up to 3 per cent). In addition when comparing the chambers it was necessary to take into account the effect of scattered radiation. Whereas the indications of the normal chamber were proportional to the intensity of the absorbed dose in air for a narrow beam of radiation and the action of scattered radiation was not considered, the indications of the extrapolation chamber (even for a narrow beam of radiation) were proportional to the strength of the absorbed dose not in the narrow, but in the wider beam of radiation close to the surface giving perceptible back scatter from the thick rear wall of the chamber.

VOL. 5 1960

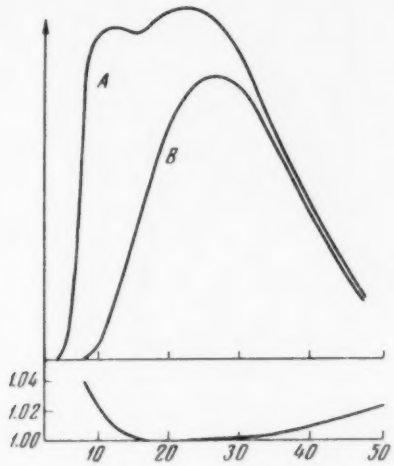


FIG. 4. Approximate distribution of intensity of energies for radiation from tube with Be window (for 50 $kV_{max}$). Abscissa, energy, kev; ordinates, intensity in arbitrary units; *A*, curve for radiation filtered with 1 mm poly methyl methacrylate (frontal wall of chamber); *B*, for radiation with additional 0·5 mm Al filter; Below, graph showing ratio of mass coefficient of true absorption of water and air in relation to energy of radiation (kev).

The value of the back scatter using the BF-70 tube in the wide beam was from 3 per cent (unfiltered radiation) to 9 per cent (0·5 mm Al filter). These values were obtained experimentally.

After introducing into the measurements all the necessary corrections, the results with the extrapolation chamber used as a "gas action" system (for $h > h_r$) and from a normal chamber agreed with an accuracy of 3 per cent (results of 12 measurements).

Fig. 5 shows the results of comparisons of the chambers. The abscissa gives the height of the clearance ($h$) in mm, the ordinates the ratio of $\frac{1}{Q}\, dI/dh$, for the extra-

polation chamber with the given material for the walls, to $I_0$ the density of ionization in the normal chamber. The axis of the abscissa, to be more accurate its ordinate, corresponds to $(1/I_0Q)(dI/dh) = 1$. In line with the above remarks, the values of $h$ at which the curves intersect this axis are equal to $h_r$. For $h > h_r$ the curves merged with the axis for here the results of the normal and extrapolation chambers after introducing the corrections mentioned above coincided. For $h < h_r$ in the transitional region the curves were somewhat above the axis (Cu, Al) or below it (graphite). In the case of the Cu chamber, with 0·5 mm Al filtered radiation, any change in $h$ within the limits of 0·4 mm $< h <$ 20 mm corresponds to the transitional region. In this case a system of "gas action" was not obtained.

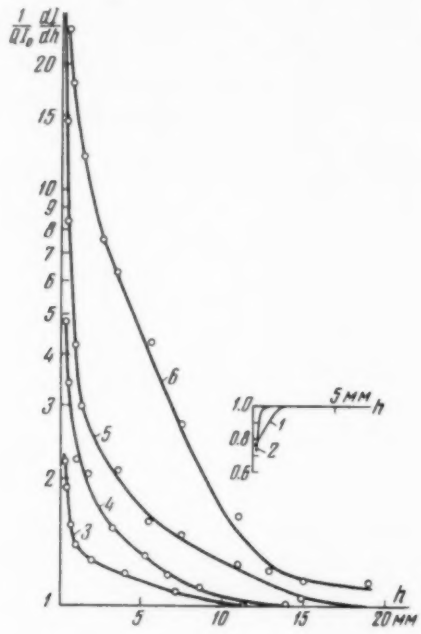


FIG. 5. Results of comparison of extrapolation chamber with normal chamber. Abscissa, height of clearance of extrapolation chamber ($h$) in mm; ordinates, magnitude $1/I_0Q$. $dI/dh$; *1*, C chamber, radiation filtered with 0·5 mm Al; *2*, C chamber, radiation without additional filter; *3*, Al chamber, radiation without additional chamber; *4*, Al chamber, radiation filtered with 0·5 mm Al; *5*, Cu chamber, radiation without additional filter; *6*, Cu chamber, radiation filtered with 0·5 mm Al.

On the basis of the results obtained, all dosimetry for radiation from the BF-70 tubes was carried out with the chamber of the usual design (graphite covered polymethyl methacrylate) at 2 mm $< h <$ 20 mm in a "gas action" system (we would note that the measurements in this mode of operating the chamber cannot in fact be called "extrapolation").

*Measurements of the dose field close to the tube window*

In measurements in the direct vicinity of the window, giving inhomogeneity of the radiation field, the chamber was used with a diaphragm of small diameter, 3–6 mm. In addition, to facilitate the achievement in the chamber of saturation conditions for all measurements close to the window, the entry diaphragm of the chamber was set at a definite and not too small distance from the frontal wall. An example will show why this was necessary. The intensity of the absorbed dose in air in the direct vicinity of the tube window in conditions of 60 $kV_{max}$, 40 mA, with a filter of 1 mm polymethyl methacrylate reaches, as shown by measurement, 730,000 rad/min, corresponding to an ionization density of about $10^4$ e.s.u./cm$^3$/sec. If the ionization chamber were placed directly under the diaphragm ($l = 0$) such a density of ionization would be within the chamber. To ensure saturation for such ionization densities and for a clearance $h$ (in the chamber) of 1 cm, it is necessary to apply a voltage in the chamber of about $10^4$ V (according to paper [10]). In practice, in carrying out the measurements only the entry diaphragm of the chamber was near the window of the tube and the actual chamber (its frontal wall) was 45 mm away from the diaphragm (i.e. $l = 45$ mm). The beam of radiation coming from the aperture of the diaphragm was strongly divergent. As photography of the beam established, the surface area exposed to it on the frontal wall of the chamber was about 400 times greater than the area of the aperture of the diaphragm of the chamber. In view of this the real ionization density on measurements in the chamber was about 400 times less than $10^4$ e.s.u./cm$^3$/sec and the voltage required for saturation in these conditions ought to be only $10^4/\sqrt{400} = 500$ V.

The strength of the absorbed dose in air, $P$ (unit of measurement, rad/min) was calculated from the formula:

$$P\,(\text{rad/min}) = 2{\cdot}88 \times 10^{-3} \frac{\omega}{\rho \cdot e} \frac{f}{Q} \frac{dI}{dh}$$

where $Q$, is the area of the entry diaphragm of the chamber corrected for transmission (see above) in cm$^2$; $dI/dh$ the slope of graph $I(h)$ to the axis $h$ in $A$/cm; $\rho$, the density of air (g/cm$^3$); $\omega$, the average energy of formation of an ion-pair (eV); e, the electronic charge; $f$, the correction factor allowing for the possible attenuation of the divergent beam of radiation in the path between diaphragm and the frontal wall of the chamber (always determined experimentally; in our measurements $1{\cdot}03 < f < 1$).

The chamber operating as a "gas action" system measures the intensity of absorbed dose in air. As was shown above, this value was quite close to the absorbed dose in water. Therefore, the method described is convenient for dosimetry or irradiation of thin water-equivalent objects. To determine the absorbed dose in materials differing from water the usual calculations are required (from the difference in the coefficients of the true absorption of the given material and air).

Fig. 6 presents the results of measurements near the window of the BF-70 tube. In Fig. 6 (A) is given the change in dose rate with position along the elongated window

of the tube at its surface. The measurements were made with a 0·25 mm Al filter. The dose rate (in air) at the centre of the window was 160,000 rad/min. The results of the measurements are denoted by small circles and the unbroken curve is the result of calculation. It was assumed for the calculation that the 26 mm long ruled focal surface of the tube lay at a distance of 13 mm from the plane of measurements, that each point of the focus emits isotropically and that absorption of radiation can be

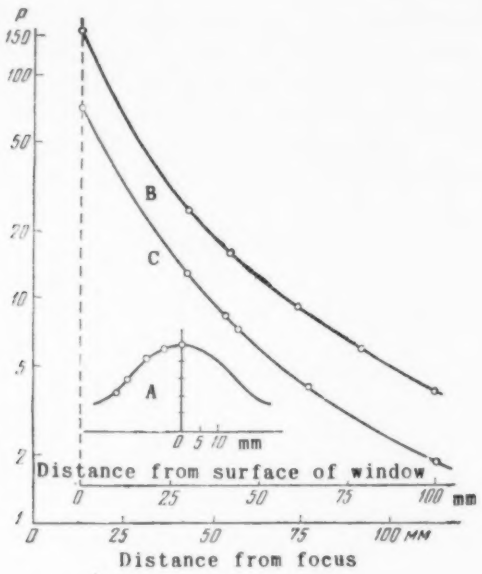


FIG. 6. Results of measurements near window of BF-70 tube. *A*, change dose rate on movement along surface of window, filter 0·25 mm Al. Abscissa, distance in mm from centre of window, ordinate, dose rate. Dose rate in air in centre of window 160,000 rad/min. *B*, decline in intensity of absorbed dose in air on with distance from the window. Abscissa, distance from surface of window or from focus of tube in mm, ordinates, dose rate in air. Upper curve *B* for radiation filtered with 0·25 mm Al, numbers along axis of ordinates in 1000 rad/min; lower curve *C* for unfiltered radiation, numbers along axis of ordinates in 10,000 rad/min.

disregarded. In Fig. 6 (B) and (C) is shown the decrease in dose rate with distance from the window (along a line perpendicular to the axis of the tube and passing through the centre of the window).

## SUMMARY

(1) The method described of a wide flat ionization chamber with variable clearance between electrodes and the diaphragm can be applied to measure the strength of the absorbed dose in air in an inhomogenous field of soft X-radiation close to the source. The method makes it possible to achieve with ease saturation conditions in the chamber with dose intensities of the order of $10^6$ r/min, and higher, for a clearance width in the chamber of 1 cm.

(2) The method makes it possible in principle by changing the depth of the chamber ($h$) to operate as a "wall action" or "gas action" system. The first system is applicable for harder radiation, the second for softer. On changing the gas pressure in the chamber simultaneous with change in $h$ one could obviously use both systems for measurements of the given radiation.

(3) Certain criteria are indicated for distinguishing wall and gas modes of operation.

In this case use can be made of: (a) the form of the graph $I(h)$ (linearity in the wall and gas regions, but with different inclination to axis $h$, curvilinearity in the transitional region); (b) the variation of the slope of the graph with the material of the walls (change in slope of the "wall" part of the graph on replacement of one material by another, invariability of the slope of the "gas" part of the graph); and (c) comparison of the results of the extrapolation chamber with those of a normal one.

(4) The design of the ionization chamber used is such that "modelling" of the conditions of measurement on radiation is simplified.

For such "modelling": (a) the measurements are made precisely in that beam of radiation (completely passing through the chamber) which falls on the object; (b) one can from the measurement estimate accurately without the introduction of any corrections, the effect of absorption and scatter of radiation on the dose absorbed in the object. As a result the accuracy of the dosimetry is raised.

This work has been concerned with the use of a flat extrapolation chamber with a diaphragm for the dosimetry only of soft X-or $\gamma$-emissions. However, a similar method may be applied for "near focus" measurements for other types of low-penetrating radiation (for example, $\alpha$ and $\beta$-radiation). Thus, the method described with a slight modification has been used by the author to measure the strength of the absorbed dose from $^{210}$Po $\alpha$-radiation over small distances from an extended source. The results of this work will be described in another article.

The author wishes to express his appreciation to Prof. Ia. L. Shekhtman for his interest, assistance and participation in discussion throughout the work.

*Translated by* A. CROZY

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## METHOD AND APPARATUS

# CERTAIN ASPECTS OF THE DOSIMETRY OF DISTRIBUTED SOURCES OF β-RADIATION*

A. M. KONONENKO and V. A. PETROV

Central Medical Radiology Research Institute of the U.S.S.R. Ministry of Health, Leningrad

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VOL
5
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THE problem of determining the dose produced by distributed sources of radiation arises whenever the action of emissi ns of incorporated radioactive substances or radioactive substances distributed outside the body is under consideration.

Determinations of the mean doses in the organism for β-ray emitters are the most practicable and widely-used me hods [1]. However, in investigating the biological action of ionizing radiations knowledge of the mean dose alone is not sufficient. It is important to be able to take into account any inhomogenous distribution of the radioactive emitters when determining the dose. It is particularly essential to know the distribution of doses at the boundary between the various media, where the variations may be considerable.

At present this problem is being tackled from various angles. Solutions for the mean dose in a layer irradiated with β-particles from a thin applicator have been given by Petrov [2, 3]. Ardashinkov and Chetverinkov [4] have devized methods for calculating the integral dose from a point source on the basis of the exponential absorbtion of the particle's energy, taking into account the finite range of the β-particle. These workers suggest the possibility of their method being used to compute the dose at points arranged on the axis of figures of rotation containing distributed β-emitters. Rossi and Ellis [5] have sought to estimate the distribution of dose from sources of β-radiation homogenously distributed in one of the boundary media. They started from the assumption that the absorption in the medium of the energy of the β-radiation from a point source is exponential and may be characterized by a definite constant coefficient of absorption. Using this approximation, Rossi and Ellis obtained a non-exponential distribution of dose at the flat interface between two homogenous semi-infinite media, in one of which the β-emitter was homogenously distributed. However, the experimental findings of Bailey reported in the same paper indicate the explicitly marked exponential character of the distribution of dose.

Of interest is the work of Loevinger [6, 7] in which he attempted to find a sufficiently accurate analytic expression for the distribution function of the absorbed energy in an homogenous medium surrounding a point source. Determination of this function would allow one, within the possible limits of integration, to solve the problem of the distribution of dose from β-emitters. However, difficulties in the way of doing this are still considerable.

There is a further possibility of calculating the distribution of dose from β-sources which does not involve the need to use the "function of point source". Instead of considering the distributed β-emitter as an integral totality of point sources, such an emitter can be regarded as the sum of distributed sources of mono-energetic electrons, the distribution of dose from them can be determined and then integrated over the spectrum of emission. Although precise integration of the β-spectrum presents great difficulties it can successfully be replaced by summation of dose from a finite number of mono-

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energetic components. Such a method has been applied by Woodard and Spiers [8] to estimate the dose produced in the tissues by electron emission coupled with X-radiation. This method also enables any existing solutions for mono-energetic emitters in the same conditions [9, 10] to be used to obtain a solution.

In the present paper we shall consider the results of some calculations of the distribution of the intensity of dose in certain simple geometric conditions.

## 1. DISTRIBUTION OF THE INTENSITY OF A DOSE FROM $\beta$-RADIATION AT A FLAT INTERFACE BETWEEN TWO SEMI-INFINITE MEDIA

The equation for the distribution of dose rate of mono-energetic corpuscular emission at the flat interface between two semi-infinite homogenous media containing distributed emitters has been defined for the special case by Munson [9] and in the more general form by Kononenko [10].

Using this equation we can approximately represent the dose rate distribution between two media — $i$ and $k$ from β-sources distributed in them in the following form:

for medium $i$

$$Pd_i = \sum_{m=1}^{m=n} E_{\beta m}\left\{N_{im} + \frac{1}{2}\left(N_{km}\frac{R_{km}}{R_{im}} - N_{im}\right)\left[1 + \frac{l}{R_{im}}\left(\ln\frac{l}{R_{im}} - 1\right)\right]\right\} \tag{1}$$

for medium $k$

$$Pd_k = \sum_{m=1}^{m=n} E_{\beta m}\left\{N_{km} + \frac{1}{2}\left(N_{im}\frac{R_{im}}{R_{km}} - N_{km}\right)\left[1 + \frac{l}{R_{km}}\left(\ln\frac{l}{R_{km}} - 1\right)\right]\right\}, \tag{2}$$

where $Pd_{i,k}$, the dose at distance $l < R_{i,k}$ from the boundary; $E_{\beta m}$, the energy of the β-particles of the mono-energetic component $m$; $N_{im}$, $N_{km}$ the number of β-particles with a given energy emitted per unit time per unit volume of medium $i$ or $k$ respectively; $R_{im}$, $R_{km}$ the range of the electrons of energy $E_{\beta m}$ in media $i$ and $k$; $R_i$ and $R_k$ the range of electrons having maximal energy; $n$, the number of approximately mono-energetic components into which the β-spectrum is divided.

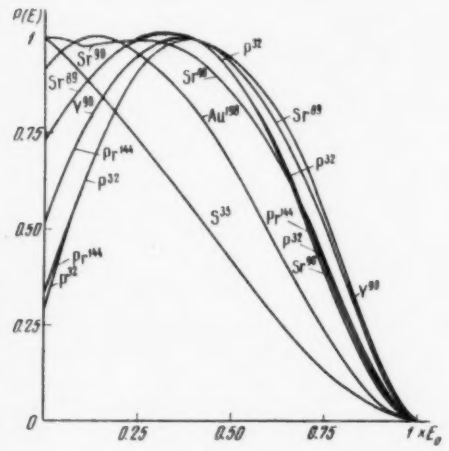


FIG. 1. β-spectra for $^{32}$P, $^{35}$S, $^{89}$Sr, $^{90}$Sr, $^{90}$Y, $^{144}$Pr and $^{198}$Au.

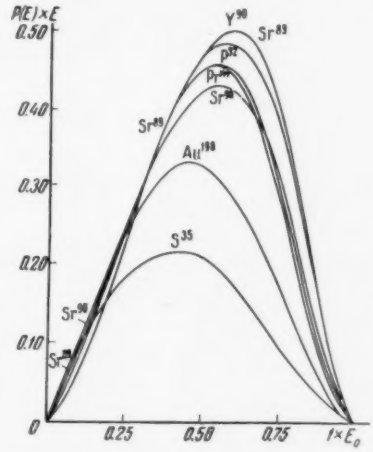


FIG. 2. β-spectrum energy distribution for $^{32}$P, $^{35}$S, $^{89}$Sr, $^{90}$Sr, $^{90}$Y, $^{144}$Pr and $^{198}$Au.

It should be noted that in the conditions where there is no redistribution of radioactive substances with time, the distribution of the dose rate and the dose is identical.

The formulae given do not take into account the scatter of electrons which leads to additiona transfer of energy from the medium of higher concentration to a medium with a lower concentration of β-emitter. Although, as will be shown below, the contribution of the scattered emission to the dose for hard and medium β-rays is not high, but failure to allow for this factor is probably one of the reasons for the divergence found between the theoretical and experimental results in the case of soft β-emissions.

To calculate the distribution of dose rate according to formulae (1) and (2) it is necessary to know the shape of the spectrum of the β-emitter. At present there are quite good tables [11] for calculating with sufficient accuracy the form of the spectrum for any allowed β-transfer.

Fig. 1. gives the β-spectra calculated by us of certain radioactive elements having the practical value:

$$^{35}S\ (E_0 = 0{\cdot}167\ \text{Mev}),\ ^{198}Au\ (E_0 = 0{\cdot}960\ \text{Mev})$$
$$^{32}P\ (E_0 = 1{\cdot}70\ \text{Mev}),\ ^{144}Pr\ (E_0 = 2{\cdot}97\ \text{Mev})$$

with the spectra of the allowed form and

$$^{90}Sr\ (E_0 = 0{\cdot}54\ \text{Mev}),\ ^{89}Sr\ (E_0 = 1{\cdot}46\ \text{Mev}),\ ^{90}Y\ (E_0 = 2{\cdot}25\ \text{Mev})$$

having the so-called α-form of β-spectra corresponding to certain forbidden transitions [12]. The abscissa gives the energy of electrons expressed in fractions of the maximal energy of the spectrum $E_0$, the ordinates, the intensity expressed in fractions of the maximal intensity. The distribution of energy over the spectrum is shown in Fig. 2.

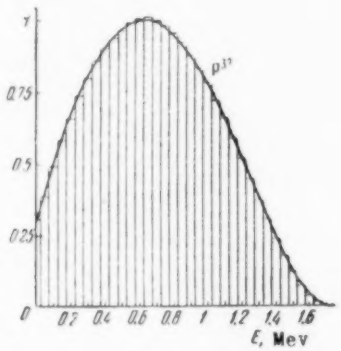


FIG. 3. $^{32}P$ spectrum represented by mono-energetic components.

Each of the calculated β-spectra was approximately represented in the form of the sum of the mono-energetic components, as shown in Fig. 3.

The difference in the energy of two adjacent components was from 3 per c.nt ($^{32}P$) to 6 per cent ($^{35}S$). But since the errors left and right of the axis of each column largely offset one another, the total error in the number of electrons and the total energy will be considerably less than the values indicated above.

A calculation was made of the distribution of dose rate at the flat interface between two semi-infinite homogenous media ($i$ and $k$), one of which contained the β-emitter, for each mono-energetic component according to the expression under the summation sign in formulae (1) and (2). Then the distribution curves obtained were summated graphically.

In Fig. 4 the unbroken lines denote the calculated distribution of dose rate for $^{32}P$, $^{35}S$, $^{89}Sr$, $^{90}Sr$, $^{90}Y$, $^{144}Pr$ and $^{198}Au$ distributed in medium $k$ in the absence of an emitter in medium $i$ (in equations (1) and (2), $N_{im} = 0$).

The abscissa depicts the distance from the boundary in fractions of the maximal depth of the penetration of electrons, the ordinates the dose intensity in fractions of its equilibrium value.

From formula (2) the equilibrium dose rate in medium $k$, containing the β-emitter, is given by:

$$Pd_k = \sum_{m=1}^{m=n} N_{km} E_{\beta m} = N_k \bar{E}_\beta , \qquad (l = R_k) \tag{3}$$

where $N_k$ is the total number of β-particles emitted per unit time per unit volume of medium $k$; $E_\beta$, the mean energy of the electrons. This value for the equilibrium dose rate is the starting point for the calculation of the distribution of dose rate in medium $k$. The values for the dose rate in the irradiated medium $i$ will depend not only on the value of the equilibrium of dose rate in the radiation-emitting medium, but also on the ratio of the stopping powers of both media. In fact, from formula (1) it follows that directly at the boundary with medium $k$, i.e. when $l = 0$, the dose rate in medium $i$ (for $N_{im} = 4$) is expressed in the following form:

$$Pd_i = \frac{1}{2} \sum_{m=1}^{m=n} N_{km} E_{\beta m} \frac{R_{km}}{R_{im}} = \frac{1}{2} N_k \bar{E}_\beta \frac{R_k}{R_i} = \frac{1}{2} Pd_k \frac{R_k}{R_i} , \tag{4}$$

since the ratio $R_{km}/R_{im} = R_k/R_i$ is for the given conditions a constant value, as it depends only on the ratio of the stopping powers of the media and not on the energy of the β-particles. Thus, the value $Pd_K \dfrac{R_k}{R_i}$ is the starting point for calculating the dose rate in medium $i$. The scale presented in Fig. 4 expresses the dose rate in medium $k$ in fractions of $Pd_K$ and in medium $i$ in fractions of $Pd_K \dfrac{R_k}{R_i}$. We shall also refer to the latter value as the equilibrium dose rate. With identical stopping power of the media $i$ and $k$ the ratio $\dfrac{R_k}{R_i} = 1$.

It follows from Fig. 4 (a) that the distribution of dose rate calculated in the irradiated medium for all the isotopes considered is exponential between a depth of 0·02 $R_i$, where the intensity of dose is 0·4 of the equilibrium dose or 80 per cent of the surface dose, and a depth of 0·4 $R_i$ where the dose rate becomes insignificant and is from 0·008 for $^{35}S$ to 0·025 of the equilibrium dose for $^{90}Y$. At depths greater than 0·5 $R_i$ the dose rate is less than 0·005 of the equilibrium dose and rapidly falls off to negligible values.

In Fig. 4(b) are plotted the experimental values obtained by Bailey and the theoretical curves of distribution of dose rate according to Rossi and Ellis (broken lines). The experimental values obtained by Bailey and also the regularity in the disposition of the experimental points for $^{32}P$ and $^{204}Tl$ are in good agreement with our theoretical curves. The small and quite admissible deviation of the experimental points for $^{32}P$ towards larger values, is probably related to the omission in our calculation of the influence of scattering. However, for $^{35}S$ with a soft β-spectrum, the theoretical curve runs far below the line passing through the experimental points. In this case, the probable cause is the low accuracy of the calculation due to the fact that the depth of penetration of soft and very soft electrons has not been adequately studied and also the omission of the influence of electron scatter.

The theoretically-calculated exponents for $^{90}Sr$, $^{198}Au$, $^{32}P$, $^{98}Sr$, $^{144}Pr$ and $^{90}Y$ and the experimental one for $^{35}S$ practically converge at the boundary at the point corresponding to 0·45 $Pd_k \dfrac{R_k}{R_i}$. We can thus express the dependence of dose rate in the irradiated medium $i$ for depths from 0·02 $R_i$ to 0·4 $R_i$ by the following formula:

$$Pd_i = 0{\cdot}45\, Pd_K \frac{R_k}{R_i} \cdot e^{-\mu l}, \tag{5}$$

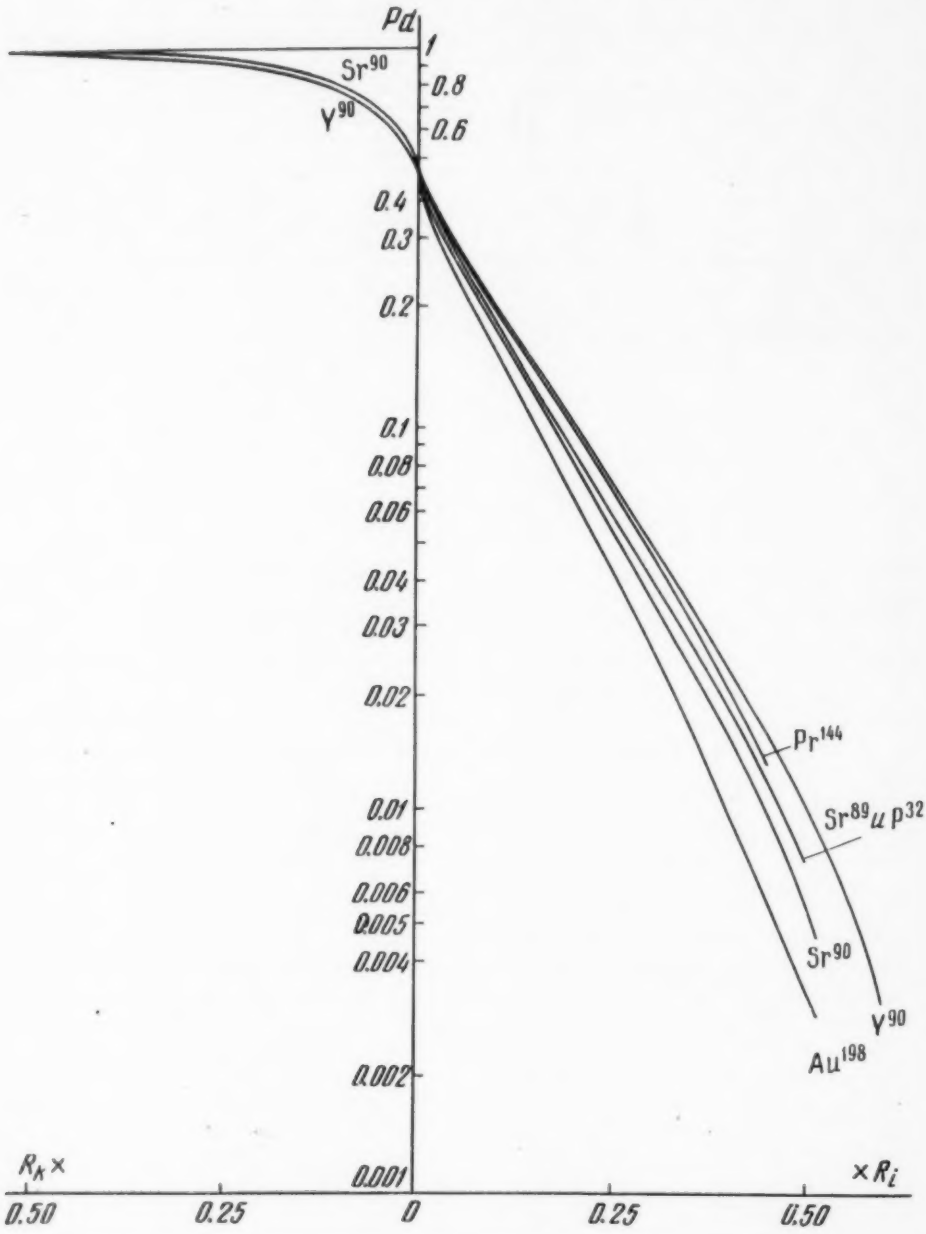


FIG. 4(a) Distribution of dose rate *Pd* as a function of distance to boundary of active inactive media for $^{32}P$, $^{89}Sr$, $^{90}Sr$, $^{90}Y$, $^{144}Pr$ and $^{198}Au$, according to the calculated data.

where $Pd_i$, is the intensity of dose in the irradiated medium $i$ at a distance $l$ from the boundary, and $\mu$ is the coefficient of absorption.

If the depth of penetration is expressed in the fractions of the range, then $\mu$ becomes a dimensionless constant and assumes the values: $^{35}S$ 10·0, $^{198}Au$ 9·1, $^{32}P$ 8·0, $^{144}Pr$ 7·4, $^{90}Sr$ 8·6, $^{90}Y$ 7·2 and $^{89}Sr$ 8·0.

The distribution of dose rate in the emitting medium $k$ for depths from 0·02 $R_k$ to $R_k$ is obviously expressed by the following formula:

$$Pd_k = Pd_K(1 - 0{\cdot}45\, e^{-\mu l}). \tag{6}$$

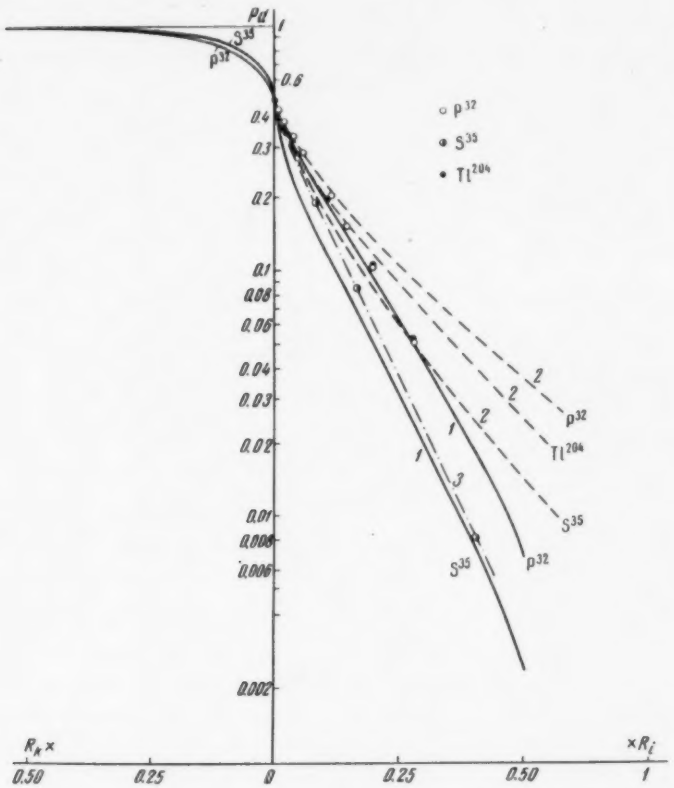


FIG. 4(b) Distribution of dose rate $Pd$ as a function of distance to boundary of active and inactive media. 1, our calculation; 2, calculation according to formulae of Rossi and Ellis [5]; 3, curve for $^{35}S$ plotted from Bailey's experimental points. Points show Bailey's experimental data [5] for the isotopes in question.

Directly at the boundary $Pd_i = 0{\cdot}5\, Pd_K \dfrac{R_k}{R_i}$ and $Pd_k = 0{\cdot}5\, Pd_K$ i.e. in the first case by a 10 per cent greater value than that obtained from formula (5); in the second by a 10 per cent lower value than that given by formula (6).

In the general case, when the same β-emitter is contained not only in medium $k$, but in medium $i$, i.e. $N_i \neq 0$, as can be readily seen from equations (1) and (2), formulae (5) and (6) assume the following form:

$$Pd_i = Pa_j + 0{\cdot}45\left(Pd_K\frac{R_k}{R_i} - Pd_j\right)e^{-\mu l} \tag{7}$$

$$(0{\cdot}02R_i < l < 0{\cdot}4\ R_i)$$

$$Pd_k = Pd_K + 0{\cdot}45\left(Pd_j\frac{R_i}{R_K} - Rd_K\right)e^{-\mu l} \tag{8}$$

$$(0{\cdot}02\ R_k < l < 0{\cdot}4\ R_k).$$

In this case, the curves in Fig. 4 refer to the second terms in formulae (7) and (8), i.e. give a value for the dose rate which must be algebraically summated with the equilibrium dose to obtain the value of the dose rate at distance $l$ from the boundary. In Fig. 4 the dose rate corresponds to the fractions of the value

$\left(Pd_K\frac{R_k}{R_i} - Pd_j\right)$ for medium $i$ and

$\left(Pd_j\frac{R_i}{R_k} - Pd_K\right)$ for medium $k$

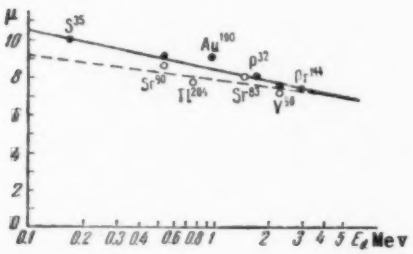


FIG. 5. Variation of absorption coefficient $\mu$ with maximum energy ($E_0$) of β-emitter. Unbroken curve, for elements with allowed β-spectrum; broken curve, for elements with certain forbidden forms of spectra.

Fig. 5 shows the dependence of the dimensionless coefficient of absorption $\mu$ on the energy of β-emission. In this case, the variation of $\mu$ with $E_0$ is obviously chiefly determined by the fact that the depth of penetration of the ele trons into the medium is not proportional to their energy.

The values of $\mu$ for the allowed form of the β-spectra ($^{35}$S, $^{32}$P, $^{144}$Pr and $^{198}$Au) and also for the allowed form calculated for $Z$ and $E_0$ corresponding to $^{90}$Sr and $^{90}$Y, are disposed along the straight line described by the equation:

$$\mu = 8{\cdot}4 - 2{\cdot}1\log E_0, \tag{9}$$

where $E_0$ is in Mev (unbroken curve)

For $^{198}$Au with the allowed form of β-spectrum, the value $\mu$ deviates by 8 per cent towards higher values from the value corresponding to the straight line. This is connected with the la ge nuclear charge of the element which softens the β-spectrum considerably (Figs. 1 and 2).

The β-spectra with the α-form are somewhat harder than the allowed spectra of the same maximal energy (Fig. 2) and have lower values of $\mu$. In this case the values obtained for $\mu$ do not differ by more than 4 per cent from the straight line described by equation:

$$\mu = 7{\cdot}9 - 1{\cdot}3\log E_0, \tag{10}$$

where $E_0$ is in Mev (the broken line in Fig. 5).

For practical purposes it is more convenient to express $\mu$ in dimensional values. Fig. 6 gives the variation of $\mu$ expressed in $cm^{-1}$ with $E_0$ for a medium of unit density. For the spectra of the allowed form the values of $\mu$ are located along a straight line described by equation:

$$\log \mu = 1{\cdot}35 - 1{\cdot}43 \log E_0 \tag{11}$$

or

$$\mu = 22{\cdot}4 E_0^{-1{\cdot}43}, \tag{11a}$$

with $E_0$ in Mev.

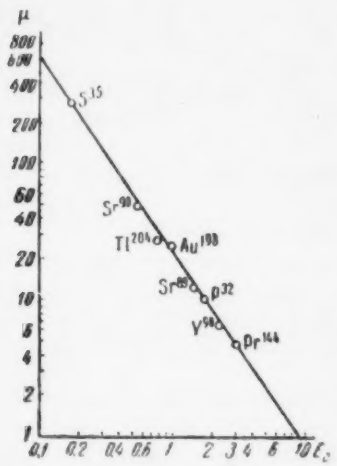


FIG. 6. Variation of absorption coefficient $\mu$ with energy of β-emitter $E$ for medium of unit density.

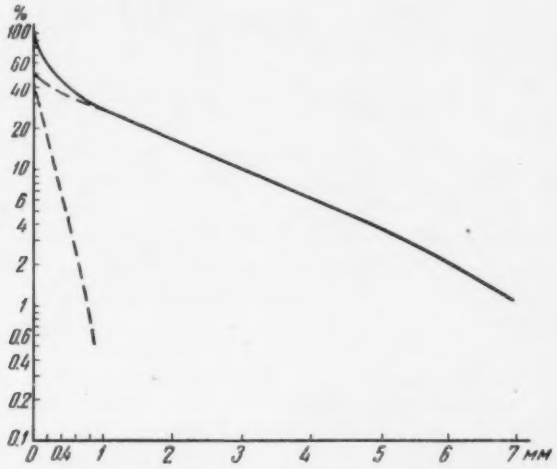


FIG. 7. Distribution of total dose from $^{90}Sr+^{90}Y$ in medium of unit density (unbroken line). Abscissa, depth of penetration; ordinate, dose.

For the β-spectra with the α-form the values of $\mu$ are somewhat less but none of points in Fig. 6 for the allowed and forbidden spectra deviate by more than 12 per cent from the values of $\mu$ corresponding to the straight line described by equation (11).

It is of interest to determine the distribution of the total dose rate from $^{90}Sr$ and $^{90}Y$ (in equilibrium) distributed in the medium. This distribution is graphically presented in Fig. 7 in percentages of the surface dose rate.

## 2. DISTRIBUTION OF THE DOSE RATE FROM β-EMISSION FROM A FLAT LAYER OF FINITE THICKNESS

The solutions for the semi-infinite media make it possible to solve the problem of the distribution of dose rate from a layer of finite thickness.

In fact, an infinitely extended layer the thickness of which is equal to $d$, can be represented as the difference between two semi-infinite media one of which is directly adjacent to the boundary, the second being separated from it by distance $d$.

Accordingly, the dose rate from the given layer in any point at distance $l$ from the boundary, can be depicted as the difference in the intensities of dose produced at the same point by the two semi-infinite media mentioned above.

In accordance with equation (5), the intensity of dose produced at a point at a distance $l$ from the boundary by the semi-infinite medium directly contiguous with the boundary, is expressed in the following form:

$$Pd_l = 0{\cdot}45\, Pd_K \frac{R_k}{R_l} e^{-\mu l}$$

The intensity of dose produced at this point by the semi-infinite medium at distance $d$ from the boundary,

$$Pd_i = 0{\cdot}45\, Pd_K \frac{R_k}{R_i} e^{-\mu(l+d)}$$

By subtracting the second from the first equation we get the following expression for the distributed intensity of dose from the layer of thickness $d$:

$$Pd_i = 0{\cdot}45\, Pd_k \frac{R_k}{R_i} e^{-\mu l}(1 - e^{-\mu d}). \tag{12}$$

It should be noted at once that the application of formula (12) is limited to the following conditions: $l > 0{\cdot}02\ R_i$, $(l+d) < 0{\cdot}4\ R_i$. It follows from these conditions that the distribution of dose rate may be determined in the interval of depths $0{\cdot}02\ R_i < l < 0{\cdot}4\ R_i - d$.

Thus, the smaller $d$, the greater the interval of depths in which the distribution of intensity of dose from the layer can be calculated. The limiting value is the value $d = 0{\cdot}38\ R_i$ at which according to formula (12) the intensity of dose can be calculated only at the point $l = 0{\cdot}02\ R_i$.

By using graphs the dose rate from the layer can be calculated for a somewhat wider interval

Despite the limitations, graphically and according to formula (12) it is possible to calculate the distribution of dose rate in the interval of the greatest practical importance and for the most important values of $d$. In fact, the values of the dose rate for the layer of thickness $d = 0{\cdot}5\ R_i$ differ from those produced by the semi-infinite medium by less than 2 per cent.

As follows from equation (12) the absorption coefficient does not vary with the thickness of the layer, i.e. both for thick and thin layers the relation $\mu \sim E_0^{-1{\cdot}43}$ applies. Loevinger [6] for thin layers gives the relation $\mu \sim E_0^{-1{\cdot}4}$. There is thus good agreement between our data and those of Loevinger.

In further work the problem of the distribution of dose from a layer of finite thickness will be the subject of more detailed consideration. It is most important to determine how far the solutions for the flat interface between two media are applicable to conditions in which the boundary between the media has a certain slope. Such an assessment may be made on the basis of the results obtained earlier [10].

It follows from the results of this work that at the spherical and cylindrical interfaces between media the dose rate of mono-energetic corpuscular emission from sources distributed in one of the media exceeds (on average) not more than 10 per cent that at the flat interface if the radius of the sphere is five times and the radius of the cylinder three times greater than the range of the particles. With a further increase in the radius of these figures the difference becomes very small.

These conclusions are obviously applicable to the solutions outlined above, since these solutions are obtained by summating the mono-energetic components. But since at depths greater than $0{\cdot}5\ R$, the dose rate is less than 2 per cent of the surface dose rate, it is probable that for β-emissions the criterion will be the ratio of the radius of the sphere or the cylinder to half the maximal range of the β-particles. Hence, it follows that the solutions for the flat interface are applicable to spherical and cylindrical interfaces with an error of less than 10 per cent if the radius of the sphere is more than 2·5 times and the radius of the cylinder more than 1·5 times greater than the maximal range of the β-particles.

Direct consideration of the distribution of the dose rate for β-emission in spherical volumes will be the subject of our next paper.

## SUMMARY

We have calculated the distribution of dose rate at a flat interface from β-emitters distributed in semi-infinite media and in a flat layer of finite thickness. The calculations were made by summating the dose rate from the mono-energetic components the totality of which approximately makes up the β-spectrum.

We have obtained the distribution of dose rate graphically for the entire important interval of the depth of the penetration of electrons.

It has been determined that the distribution of dose rate of β-emission for semi-infinite medium and flat layers over a considerable interval of depths is of an exponential nature. Simple formulae were accordingly derived for calculating the distribution of dose rate of β-emission in the conditions considered.

The results are in agreement with the experimental data of Bailey [5] and the data presented by Loevinger [6, 7].

*Translated by* A. CROZY

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* There is a translation into English of this work.

# APPLICATION OF CENTRIMETRE RADIO WAVES FOR NON-CONTACT RECORDING OF CHANGES IN VOLUME OF BIOLOGICAL SPECIMENS*


Iu. Ye. MOSKALENKO

Sechenov Institute of Evolutionary Physiology, U.S.S.R. Academy of Sciences, Leningrad


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THE haracteristics of the distribution of radio waves in waveguide make it possible to devize a non-contact method of recording the changes in volume of isolated preparations of small dimensions (isolated organs of embryos, individual parts of the organs of adult animals, e.g. the frog heart, muscles of insects and crustacea, single muscle fibres of vertebrates, etc.).

It is known from the theory and practice of ultra-high frequencies, that any object within a waveguide decreases the flux of electromagnetic energy on account of its absorption. The absorption depends

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on the difference between the electric conductivity and the specific inductive capacitance of the medium filling the waveguide and the object. Consequently, an object placed in a waveguide is an absorbent of electromagnetic energy.

To carry out non-contact recording of changes in volume by the method of ultra-high frequency electroplethysmography it is necessary to find a means of placing the test object in the waveguide and of selecting the basic electrical characteristics of the radiowaves used — wavelengths and intensity of the power flux.

In the rectangular waveguide with a wave of the $TE_{01}$ type the test object can be positioned through the slit which is located parallel to the faces of the wide wall of the waveguide and perpendicular to the faces of its narrow wall. Through such slits the test object can be placed at a node of the electrical field.

The sensitivity of the method of ultra-high frequency electroplethysmography depends on the shape of the test object and its orientation in the waveguide in view of the fact that the difference in linear dimensions of the object along the different axes exerts a non-uniform influence on the drop in voltage of the detector.

Because of variation of sensitivity of ultra-high frequency electroplethysmography with orientation of the test object recording of the change in the volume of its separate parts is possible. For this purpose the test object is orientated in the waveguide in such a way that the change in the area of its projection (preferably along the $X$ axis) is caused by that part of the object for which the change in volume is to be recorded.

Together with the choice of method for placing the test object in the waveguide, an important condition for successful recording of changes in volume is the determination of the electrical characteristics of the ultra-high frequency electroplethysmograph — working wavelength and magnitude of the power flux in the waveguide. Determination of these characteristics by calculation is difficult.

The choice of working wavelength depends on the size of the test object since the wavelength determines the area of cross section of the waveguide and the permissible dimensions of the aperture in its walls intended for introducing the test object. The principal determining magnitude is the size of the waveguide along the $X$ axis ($X$ axis is parallel to the wide wall of the waveguide).

From practical work on absorbing attenuators it follows that the maximum dimensions of the absorbent along the $X$ axis must be about $\lambda/10$. Objects, the dimensions of which about the $X$ axis are in excess of this value, cause a considerable increase in the reflected power and consequently a rise in the standing wave coefficient, which may effect the output of the generator while smaller dimensions may reduce the sensitivity of the method.

The dependence of wavelength on the dimensions of the object about the $Y$ and $Z$ axes is less critical and to ensure that they influence the absorbed power, the dimensions of the test object (or its pertinent part) along the $Y$ axis must not be greater than the narrow wall of the waveguide, i.e. $\sim \lambda/4$ and its dimensions about the $Z$ axis not greater than $\lambda/2$.

The second important characteristic of ultra-high frequency electroplethysmography is the value of the power flux in the waveguide on which depends the sensitivity of the method and the degree of noise. The biological effect of the energy of the electromagnetic field on the test object also depends on the magnitude of the power flux. From this point of view, the maximum permissible value of the power flux must be below the threshold for the biological effect and its choice is based on an analysis of the mechanisms of the action of the electromagnetic field on the organism. In the region of ultra-high frequency the biological effect of the energy of the electromagnetic field is expressed in a thermal and a specific effect [1, 2]. According to the data of many workers [3], the thermal effect is observed at a power flux of 0·01 W/cm$^2$ and higher. Although the thermal effect of centrimetre radiowaves on the organism has been comparatively well studied, data on the specific effect of this form of energy are very meagre.

Nevertheless on the basis of personal observations [3] it may be accepted that an energy flux with a power of less than $10^{-3}$ W/cm$^2$ does not exert a detectable biological effect. Bearing in mind that not all the absorbed power is scattered by the test object, and part of it is emitted (since the object

acts as rod antenna) one can adopt a maximum permissible value of power flux of 1 mW in the waveguide.

To enhance the sensitivity of the method the power flux in the waveguide can be increased by charging to a pulsed generator, however, in this case the mean value of the power flux must not exceed 1 mW.

Two series of experimental checks on the theoretical postulates and practical potentialities of ultra-high frequency electroplethysmography were carried out. The first series of experiments was carried out on physical models aimed at determining the optimum conditions for non-contact recording of changes in volume and also the limits of sensitivity of the method. In the second experimental series, the aim of which was to determine the practical possibilities of ultra-high frequency electroplethysmography the objects under observation were the isolated organs of a frog (*Rana temporaria*, the heart, skeletal musculature) and also the isolated heart of tadpoles in the 28th–30th stage of development.

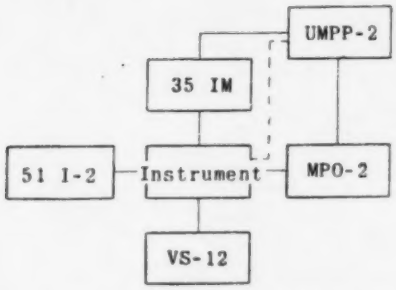


FIG. 1. Block diagram of the experimental device for ultra-high frequency electroplethysmography.

For objects of such a size the wavelength selected was about 3 cm (frequency of the order of 10,000 Mc/s). The test object was placed in the rectangular waveguide through an aperture of elliptical shape (the axes of the ellipse were 3 and 5 mm) in the centre of the wide wall of the waveguide of section 22×12 mm. The major axis of the aperture was orientated parallel to the faces of the waveguide. The waveguide was fed by a K-20 klystron type generator operating continuously. A type 35-I wavemeter was used as detector chamber. The rectified signal after amplification by a UIPP-2 instrument was recorded on a MPO-2 oscillograph (Fig. 2).

To compare the electroplethysmogram with other physiological characteristics, a waveguide section was mounted in the instrument which permitted recording of the isometric voltage in the test object and its biopotentials in response to stimulation with a single square pulse. To demonstrate the biological effect of the energy of the electromagnetic field on non-contact recording of changes in volume there was a second channel in the apparatus recording the same characteristics in a control preparation not irradiated by the energy of the u.h.f. electromagnetic field. To ensure the normal functioning of the test objects in the device there is provision for a system of moistening the preparation with nutrient solution and a system for removing the spent nutrient fluid.

The electrical arrangement of the apparatus consisted of a two-channel photoelectric recording device of the isometric voltage, an electron stimulator unit and a centimetre radiowave generator (Fig. 2).

The principle of action of the photoelectric recording device has been described previously [3].

The first series of experiments was carried out on a model consisting of a rubber tube 20 cm in length and 2 mm in diameter. The lumen of the tube was filled with 1% BaCl; one of its ends was hermetically sealed and the other connected to a rubber balloon which when pressed inflated the tube and increased its diameter to 3 mm. The tube was passed through the aperture in the waveguide and its free end was placed in a measuring burette; the change of volume, of the part of the tube in the waveguide, was estimated from the movement of the meniscus of the fluid in the burette.

The experiments on the model showed a relation between the volume of the tube and the load voltage of the detector and also enabled us to define the limits of sensitivity of ultra-high frequency electroplethysmography depending on the ratio of the diameter of the aperture in the waveguide to the dimensions (about the $X$ axis) of the test object (Fig. 3). In addition, the relationship between the attenuation introduced and the sensitivity of the method was determined.

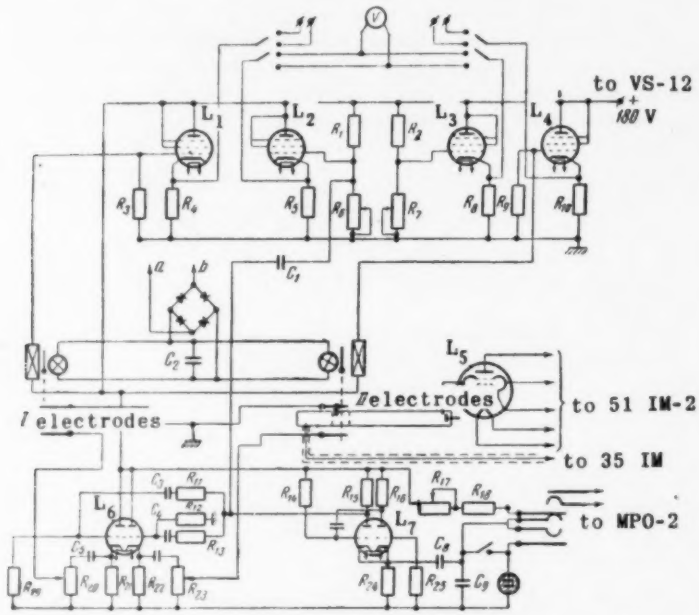


FIG. 2. Scheme of the electrical apparatus. $R_1$, $R_2$ — 200 kΩ; $R_3$, $R_9$ — 20 kΩ; $R_4$, $R_5$, $R_8$, $R_{10}$ — 2·4 kΩ; $R_6$, $R_7$ — 100 kΩ; $R_{11}$, $R_{13}$ — 50 kΩ; $R_{12}$, $R_{19}$ — 0·5 MΩ; $R_{14}$ — 1·5 MΩ; $R_{15}$, $R_{16}$ — 100 kΩ; $R_{17}$ — 4·0 MΩ; $R_{18}$ — 200 kΩ; $R_{20}$, $R_{23}$ — 22 kΩ; $R_{21}$, $R_{22}$ — 50 kΩ; $R_{24}$ — 5 kΩ; $R_{25}$ — 0·5 MΩ; $C_1$ — 1000 μμF; $C_2$ — 20 μF; 20 V; $C_3$, $C_4$ — 0·1 μF; $C_5$, $C_6$ — 0·25 μF; $C_7$ — 1000 μμF; $C_8$ — 5000 μμF; $C_9$ — 2 μF; $L_1$, $L_2$, $L_3$, $L_4$, — 6P9; $L_5$ — K-20; $L_6$, $L_7$, — 6N8S.

The maximum sensitivity was observed at a level of attenuation of 5–6 db; the dimensions of the object about the $X$ axis must be about 10 per cent of the wide wall of the waveguide.

However, with a diameter of the test object ranging from 5 to 25 per cent of the wide wall of the waveguide, the sensitivity of ultra-high frequency electroplethysmography does not fall by more than 40 per cent of the maximum.

After experimentally checking the possibility of recording the change in small volumes, a second series of experiments was on biological objects.

Fig. 4 shows a record of changes in the volume of the various parts of the heart achieved by moving the object in the waveguide along the $Y$ axis (Fig. 4 (a), (b), (c)). These records were made at a level of sensitivity of the apparatus equal to 10 per cent of the maximum. The attenuation introduced by the test object into the waveguide was about 2–3 db.

Records of the changes in the volume of the isolated frog sartorius muscle in response to a single stimulus are shown in Fig. 4(d). The attenuation introduced by the muscle into the waveguide was 1·5 db.

The standard centrimetre apparatus permits non-contact recording of the change in volume or movement of larger biological objects (the dimensions of which are equal, or even greater, than the wavelength) if it is undesirable for one reason or another to attach a recording device to them.

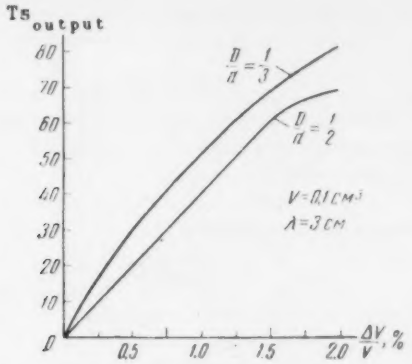


FIG. 3. Sensitivity of ultra-high frequency electroplethysmography for various diameters of aperture in wide wall of waveguide.

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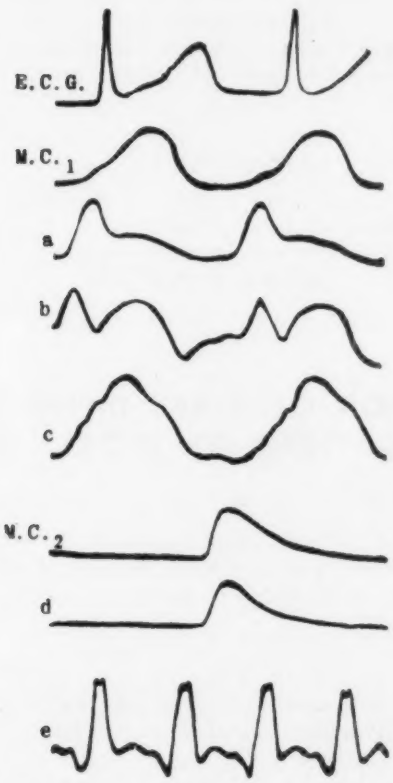


FIG. 4. Examples of non-contact recording of changes in volume of biological specimens. *a*, *b*, *c*, isolated frog heart; *d*, isolated frog muscle; *e*, isolated rabbit heart; E.C.G. and $MC_1$, electrocardiogram and mechanical contraction of test frog heart; $MC_2$ mechanical contraction of muscle.

In this case the test object may be placed between the two horn antenna, the distance between which may be 2 cm or more. Thus the contractions of the isolated rabbit heart perfused with nutrient fluid were recorded (Fig. 4 (e)).

The data presented above are merely examples, confirming the possibility of non-contact recording of the changes in volume and of the movements in an electromagnetic field of centrimetre radiowaves.

In reality, the limits of application of ultra-high frequency electroplethysmography are considerably wider. For each particular experiment it is necessary to select, on the basis of the theoretical considerations noted above, the most convenient characteristics of the electromagnetic field and the method of placing the test object in the waveguide.

## SUMMARY

(1) On the basis of the distribution of centimetric radiowaves in a waveguide it is possible to devize a non-contact method of recording changes in the volume of biological objects with an accuracy of up to 0·5 per cent — ultra-high frequency electroplethysmography.

(2) The principle of the method of ultra-high frequency electroplethysmography is based on recording the attenuation of the power flux in the waveguide introduced by the test object. The value of the attenuation introduced is determined by the area of projection of the test object on the plane of the smallest wall of the waveguide. The method proposed has the greatest sensitivity when the test objects are of a size equal to 1/10 of the wavelength.

(3) Decrease in the working wavelength down to millimetre radiowaves opens the way to evolving methods of recording movement and changes in the volume of biological objects of microscopic proportions.

*Translated by* A. CROZY

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# APPLICATION OF X-RAY DIFFRACTION TO INVESTIGATIONS ON FROZEN BLOOD*

F. R. VINOGRAD-FINKEL', L. L. RAZUMOVA and S. N. KUDRIASHOVA

Central Order of Lenin Blood Transfusion Institute, Moscow
Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow
Faculty of Fundamental Biology of the Lomonosov State University, Moscow

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BLOOD transfusion is now a widely-used radical therapeutic method and for its wide use medical institutions require stores of blood which retain full biological properties and therapeutic activity for lengthy periods of time.

Over many years, in the specialized institutions of our country and abroad research has been going on into the theoretical bases of the preservation of blood (see the collection of "Modern Problems of Haematology and Blood Transfusion" since 1930).

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It has been established that the blood cells — erythrocytes — remain viable in blood kept for a short time outside the body in aseptic conditions. To keep blood for a lengthy period in a high-grade state, conditions must be created which ensure the maintenance of the cells' metabolic processes at a definite level.

Use is made in storing blood of preservative solutions incorporating in addition to anticoagulant and antiseptics, glucose as the substrate for glycolysis and sucrose for stabilization of the cell structure.

Inhibition of the metabolic processes in the cells at definite temperature conditions (+4 – +5°) contributes to the prolonged keeping of preserved blood.

However, even in these conditions the continuing enzymatic processes in the blood lead to ageing of the cells and their gradual death, which limits the time the blood can be kept.

For maximum inhibition of the enzymatic processes in preserved blood in our country and abroad (in England, the U.S.A. etc.) research is being carried on into the freezing of blood and its storage in such a state at sub-zero temperatures.

The chief difficulty is that erythrocytes are easily destroyed on freezing blood.

There are two main ideas on the causes of the damage to the erythrocytes on freezing. Thus, there is the widely held view that the main factor damaging the erythrocytes on freezing of blood is the ice crystals which mechanically disrupt the cell structure.

However, Lovelock [1] in studying the sensitivity of the erythrocytes to high concentrations of salts postulated that the primary factor of damage was the concentrated salt solution formed in the blood as the water freezes out. Concentrated solutions of salts cause chemical denaturation of the cell colloids.

Damage to the erythrocytes on freezing of blood can to some extent be prevented by including in the preservative solutions such substances as glycerol, ethanol, certain colloid substances etc.

The mechanism of the protective action of these substances on blood cells has received little study. Pokrovskii [2] observed that as a result of the inclusion of glucose, alcohols and salts in the preservative, the ice crystals in the blood arrange themselves in groups with the undamaged erythrocytes located between them. Lovelock [1] has shown that a protective action is exerted by substances which effectively bind water and are capable of penetrating the erythrocyte cell membrane. The result of penetration of these substances into the cells is an increased amount of water but no formation of concentrated salt solution. Lusena [3] has demonstrated the presence of a retarding influence of glycerol, proteins and sugars on the rate of spread of the ice front in the red cell suspensions. The effect of retarding crystallization is that non-freezing zones in which the erythrocytes are protected from the mechanical action of the ice crystals are maintained in the suspension.

Soviet and foreign investigators (Vinograd-Finkel', Ginzberg [4], Mollison [5] *et al.*) have now worked out special preservative solutions which to a greater or lesser degree protect the blood cells on freezing and subsequent storage at temperatures below zero. It was natural to assume from the existing ideas on the mechanisms of the action of the protective substances on the blood that the preservatives chosen should prevent the formation in the blood of large ice crystals and concentrated solutions of salts which damage the erythrocytes. The crystallinity of the substances may be assessed by the X-ray diffraction technique [6, 7]. We applied X-ray diffraction to illustrate the action of the various preservatives on the character of crystallization in the blood on freezing.

In the literature, the problem of the application of X-ray analysis to investigate frozen blood has not been discussed. However, since whole blood contains about 80 per cent water and the X-ray diffraction pattern of liquid blood coincides with that of water (the sole reflexion is a halo with an interval of about 3·5 Å), it is necessary in studies on the freezing of blood to bear in mind certain data of the X-ray investigations on frozen water.

According to a number of workers [8, 9], there exist various structural modifications of ice which can be detected in certain conditions. On rapid freezing of small volumes of water at temperatures higher than —100° a hexagonal modification of ice is formed; freezing at temperatures between —100° and —120° leads to the co-existence of hexagonal and cubic modifications; on freezing at —120° and

lower there occurs cubic modification and at temperatures between —140° and —160° the dimensions of the crystals are of the order $10^{-7}$ cm, this state of water being regarded as vitreous ice.

On crystallization on orientated ground surfaces, the phenomenon of epitaxy of ice is observed, i.e. a definite orientation of the crystals formed, the latter being determined by the structure of the ground surface [10].

When investigating the structure of frozen blood certain peculiarities in its crystallization can be expected (as compared with water) associated with the colloidal state and the submicroscopic structure of blood.

## METHODS

For the X-ray analysis of frozen blood a low temperature device was used similar to that proposed by Umanskii *et al.* [11].

Fig. 1 depicts the block diagram of the arrangement. Freezing of the blood and maintenance of a given temperature during X-ray photography was carried out by the fumes of liquid nitrogen in a polymethyl methacrylate tube of the special apparatus within the X-ray chamber. Fig. 2 gives a photograph and sketch of the apparatus.*

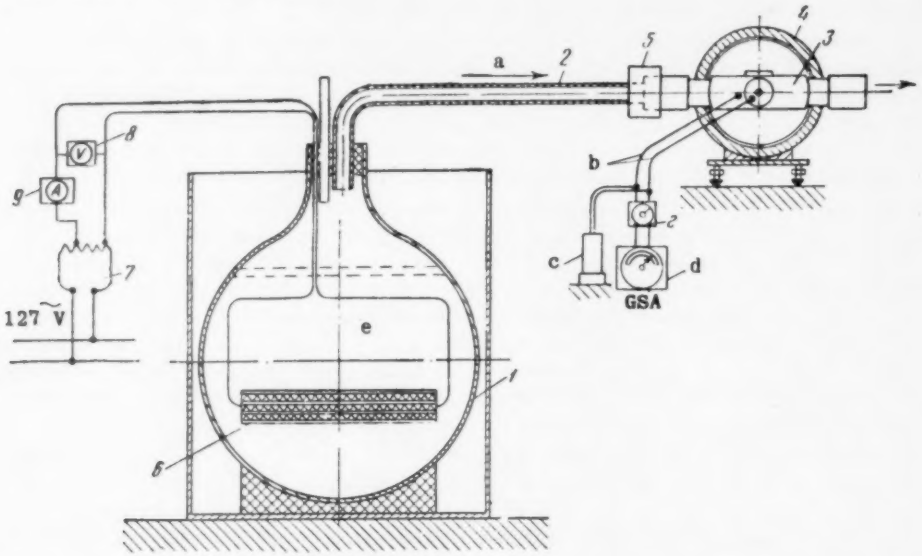


FIG. 1. Diagram of apparatus for X-ray analysis of frozen blood. 1, Dewar vessel; 2, Dewar tube; 3, freezing chamber; 4, X-ray chamber; 5, connecting tube; 6, electrical heater; 7, potentiometer; 8, voltmeter; 9, ammeter; 10, GSA galvanometer.

The tube for freezing was secured in the closed X-ray chamber by two stoppers. The sleeve of one stopper was connected to a Dewar flask through which the nitrogen fumes were fed.

Through a special aperture in the upper part of the freezing tube a small sample holder of blood was inserted in the tube. The sample holder was a square polymethyl methacrylate frame with a round hole in it, glued on each side was a fine film giving negligible scatter of the X-rays. On one side, the aperture of the frame was sealed not to the very top so that a pocket was formed which was filled with

* The design of the device for study of blood in a low temperature X-ray chamber was proposed by A. L. Lebyedev whom we wish to thank.

blood from a pipette. The thickness of the layer of the blood in the holder was 0·8 mm along the path of the X-rays.

In the opposite walls of the polymethyl methacrylate tube facing the X-ray beam there were two apertures. One — the round one — for the entry of X-rays, the other — cone-like in shape — for the exit of the X-rays dispersed in the sample. The first aperture was sealed with the same film which formed the wall of the sample holder; the second aperture was not sealed so that the nitrogen fumes freely passed into the X-ray chamber rapidly producing in it uniform temperature conditions.

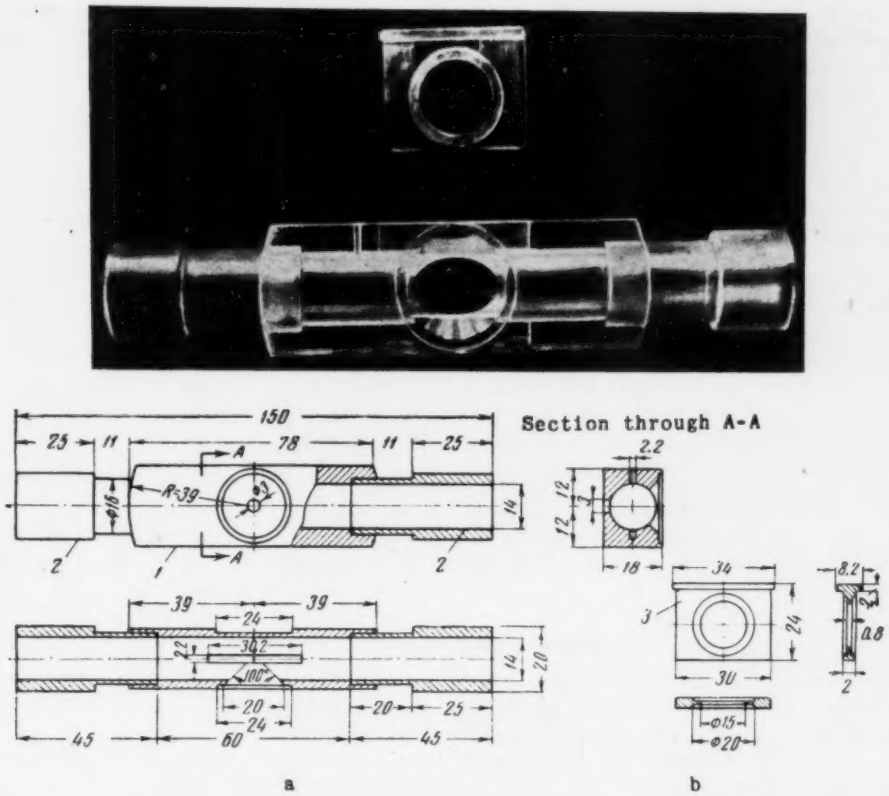


FIG. 2. Device for freezing blood in X-ray chamber and taking of X-ray pictures and the sample holder *a*, drawing of device; *b*, sample holder.

The scattered X-rays were recorded photographically. Copper $K_\alpha$ radiation was used with a specimen to film distance of about 26 mm and an exposure time of 2 hr. We obtained more than 50 X-ray photographs and up to 6 repeat exposures were taken in the same conditions of freezing.

The temperature of the nitrogen in the freezing tube and the change in temperature of the blood during freezing were recorded by copper-constantan thermocouples and galvanometers. One thermocouple was placed directly in the blood, the other in the flow of nitrogen in the polymethyl methacrylate tube near the sample holder.

Two systems of freezing the blood were employed. In the first, the sample holder containing the blood was initially placed in the tube, the whole chamber dried with silica gel and then nitrogen fumes

passed through the tube. An excess of liquid nitrogen fumes was first supplied to rapidly achieve the necessary temperature. When the given temperature was reached the fume supply was cut off. The difference in the second mode of freezing was that the blood was placed in the previously cooled chamber (at the temperature of the experiment) after passage of the fumes. The condensation of the water vapour in the sample was avoided by first blowing liquid nitrogen fumes through the freezing tube.

## BLOOD INVESTIGATED

The investigations were made on the blood samples of donors prepared in the Central Order of Lenin Blood Transfusion Institute (COLBTI) with the use of three preservatives which to varying degrees ensure the keeping of the erythrocytes on freezing.

The main results of the investigations are given in Table 1.

TABLE 1

| Blood investigated | Composition of preservative solution | Conditions of storage of blood | Suitability for transfusion after defreezing of blood |
|---|---|---|---|
| No. 7 COLBTI | Glucose-citrate solution | + 4, + 6° | Erythrocytes almost completely destroyed. Not suitable for transfusion |
| No. 11 COLBTI | Complex preservative incorporating besides sodium citrate glucose, sucrose, ethanol and polyglucin | Not frozen until —8°; stored in in frozen state at —15° | Suitable for transfusion purposes |
| M | Citrated-glycerol solution (formulated in in England by Mollison) | Stored in frozen state at —20° and lower | Suitable for tranfusion purposes after repeat washing off of glycerol and citrate |

## RESULTS OF X-RAY ANALYSIS

Before undertaking the investigation of the influence of the various preservatives on the freezing of blood, it was necessary to establish the character and degree of dependence of blood crystallization on the rate of freezing and other features of the freezing process.

To find the relation between crystallization of blood and the rate of freezing X-ray diffraction photographs were obtained of samples of blood "No. 7 COLBTI" frozen at different rates and cooled to —15°.

The X-ray pattern of the "No. 7 COLBTI" blood sample frozen slowly with a temperature gradient of not more than 2 degrees per min (Fig. 3 (a)) is similar to that for macrocrystalline substances. The X-ray pattern of water frozen in the same conditions was much the same. The similarity in the diffraction pattern of Fig. 3 (a) with the patterns observed for samples with crystal dimensions of not less than tenths of a millimeter, indicates the macrocrystalline structure of the blood frozen in this way.

In the X-ray patterns of samples of "No. 7 COLBTI" blood frozen quickly (temperature gradient from 5 to 20° per min) there were concentric rings consisting of homogenous small points (Fig. 3 (b) and (c)). From the diameter of the rings we determined the spacings of the structure which coincided with the periods for the hexagonal modification of ice [12]. The presence of rings and the small size of the points in them indicate the smaller size of the crystals in the samples investigated.

The X-ray patterns in Fig. 3 (b) and (c) differ in the character of the point array. In Fig. 3 (b) the points are uniformly arranged on the rings which is indicative of the disorderly orientation of the

crystals formed. In Fig. 3 (c), the points are arranged non-uniformly on the rings (packing through 60°), which indicates the presence of a certain predominant orientation of the crystals — the texture.

Since preliminary X-ray analysis of the film of the sample holder showed the presence of texture of the film, it may be considered that the textured crystallization was the result of epitaxy of the ice on the film — the ground surface — in conditions favouring this.

The features of the process of crystallization in both cases are expressed in the temperature curves of freezing obtained by us. In curve Fig. 3 (e) corresponding to the textured X-ray pattern, the temperature curve shows a steep drop, indicating intense and uniform freezing of the blood without supercooling.

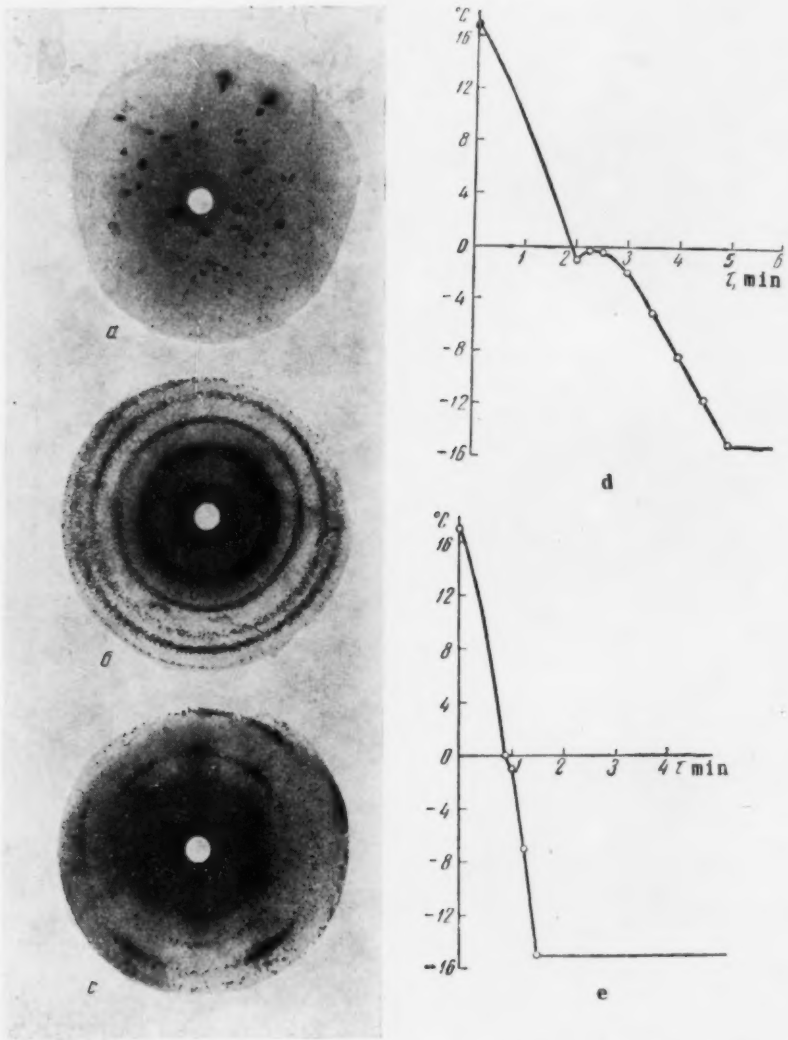


FIG. 3. (*a*) X-ray photograph of blood sample No. 7 COLBTI frozen at the rate of 2° per min. (*b*), (*c*), frozen in conditions depicted in curves (*d*) and (*e*) respectively.

It is obvious in this case that the ice front passed from the surface layers of the blood adjacent to the cooled film deep into the interior.

The curve in Fig. 3 (d) shows a temperature jump pointing to crystallization from the supercooled state. The absence in this case of crystallization texture is to be explained by the simultaneous crystallization of the entire volume of blood.

Thus, the results of the first series of investigations point to the dependence of the character of crystallization of blood (crystal size and orientation) on the speed of freezing, on the structure of the ground surface and on supercooling. This must be borne in mind in studying the relation between crystallization of blood and the composition of the preservatives.

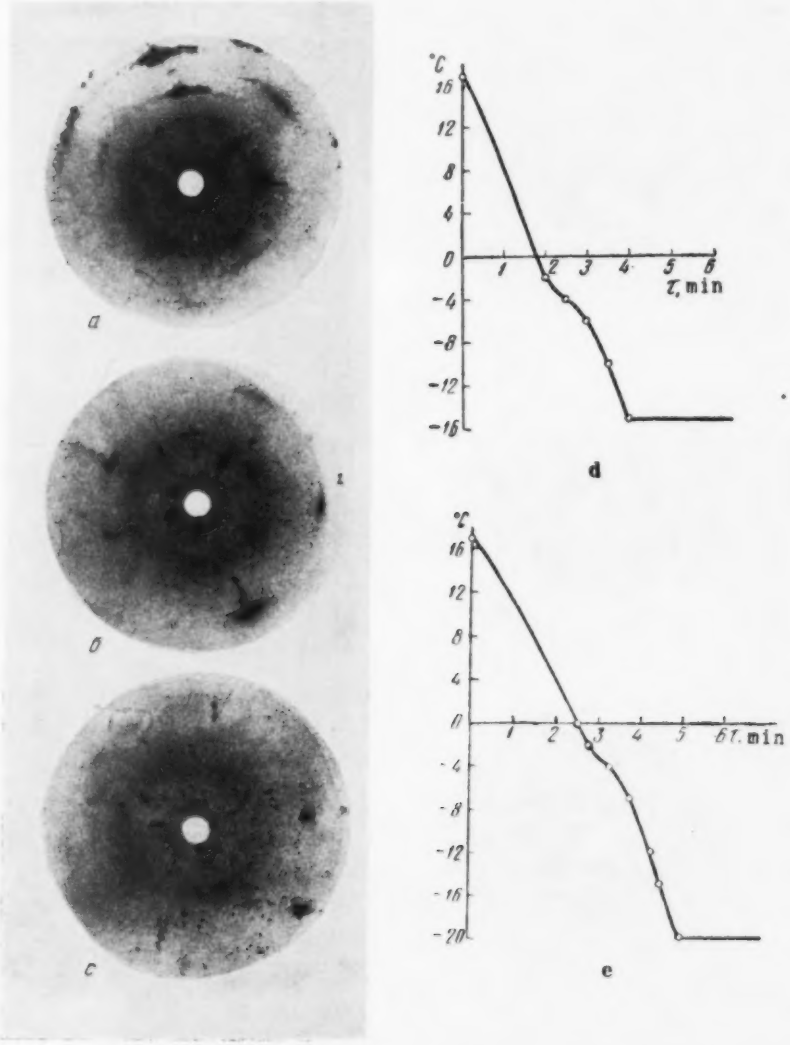


FIG. 4. X-ray photographs of No. 11 COLBTI. (*a*) and M (*b*) blood frozen in similar conditions corresponding to curves (*d*) and (*e*). (*c*) X-ray photograph of M blood frozen more slowly.

Study of the importance of the composition of the preservatives for the process of crystallization of blood was started on samples of "No. 11 COLBTI" and "M" blood. The samples were frozen in practically the same temperature conditions as for "No. 7 COLBTI" blood giving the X-ray photograph of the type depicted in Fig. 3 (c). As the curves in Fig. 4 (d) and (e) show, the rate of cooling of both samples (No. 11 COLBTI and M) was identical with a temperature gradient of about 7–8° per min. The X-ray photographs of "No. 11 COLBTI" blood were taken at —15°, of "M" blood at —20°. The first thing to be noted is the similarity in the X-ray photographs obtained for "No. 11 COLBTI" blood and "M" blood (Fig. 4 (a) and (b)) suggestive of the same type of crystallization for the investigated samples. In these X-ray photographs, as in that for "No. 7 COLBTI" blood (Fig. 3 (c)), there are textured rings consisting however of smaller points. It follows from this that the crystal size in the blood samples "No. 11 COLBTI" and "M" was smaller (of the order of several tenths of a micron) than in the blood samples "No. 7 COLBTI" frozen in the same conditions. The few large spots irregularly distributed on the individual X-ray photographs indicate the presence of one or two relatively coarse crystals (not less than tenths of a millimetre) in the volume of blood within the X-ray beam. The centrally-situated base ring in the X-ray photograph has a diffuse background indicating the presence in the blood samples of an amorphous phase in addition to the crystalline one.

OL.
5
960

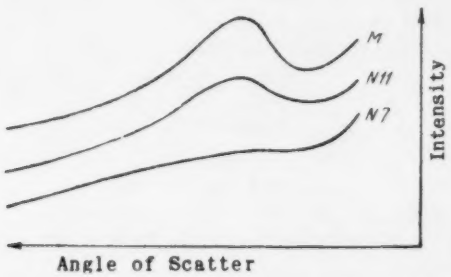


FIG. 5. Averaged microphotometric curves of radial directions of X-ray diffraction patterns of No. 7, and No. 11 COLBTI and M blood frozen in conditions shown in Fig. 4 (*d*) and (*e*) respectively.

The averaged microphotometric curves in Fig. 5 of the radial directions of the X-ray diffraction patterns of "No. 7 COLBTI" "No. 11 COLBTI" and "M" blood illustrate the differences in the character of the inner ring of these X-ray patterns. The absence of a diffuse background in the inner ring for "No. 7 COLBTI" blood suggest the absence of an amorphous phase with the presence of a continuous halo (consequently of an amorphous phase) for the blood samples "M" and "No. 11 COLBTI".

At slower rate of cooling of samples "No. 11 COLBTI" and "M" an amorphous phase was also present, but the size of the crystals formed was greater (the spots on the radiograph are larger) (Fig. 4 (c)).

Thus, as a result of an X-ray analysis of frozen blood it was found that the character of crystallization in the frozen blood is dependent on the rate of freezing, the presence of a textured base surface and supercooling. For samples of blood prepared with various preservatives and with identical rate of freezing, other conditions being equal, the factor determining the character of crystallization of the blood is the composition of the preservative.

The similarity found in the character of crystallization in the samples of blood prepared with the preservative "No. 11 COLBTI" and with citrated glycerol solution ("M" blood) suggests that the mechanism of action of this solution on the crystallization of blood is similar to the action of the complex of substances forming part of the "No. 11 COLBTI" blood solution (polyglucin, ethanol, sucrose, glucose, etc.).

The smaller size of the ice crystals formed in the blood prepared with these preservatives than in blood prepared with the glucose-citrate solution is an indication that these preservatives prevent the formation of coarse ice crystals in the blood.

The comparison of the results of X-ray analysis of "No. 11 COLBTI" and "M" frozen blood with the findings on the state of preservation of the blood cells after defreezing [4] permits us to conclude that the presence of the amorphous phase and the small size of the ice crystals in the blood prepared with these preservatives determine the integrity of the erythrocytes on freezing.

## SUMMARY

In order to bring out the character of crystallization of the blood on freezing which significantly affects the integrity of the blood cells and consequently the preservation of the therapeutic powers of the blood stored in the frozen state, low temperature X-ray diffraction techniques were applied as modified for the study of freezing of blood in various conditions.

This method helped to reveal the dependence of the character of the crystallization of blood on the composition of the preservative, the rate of freezing, the presence of a textured ground surface and supercooling.

The results indicate that the protective action of the preservative "No. 11 COLBTI" and citrated glycerol on the erythrocytes on freezing of blood is associated with the presence in the frozen blood of an amorphous phase and ice crystals of small size.

*Translated by* A. CROZY

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OL.
5
960

# LETTERS TO THE EDITORS

## EFFECT OF OXYGEN ON ELECTRON SPIN RESONANCE SPECTRA OF LYOPHILIZED TISSUES*

G. K. MOROZOVA and L. A. BILUMENFEL'D

Institute of Chemical Physics, U.S.S.R. Academy of Sciences, Moscow

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COMMONER, Townsend and Pake [1] detected narrow electron spin resonance (e.s.r.) signals in specimens of lyophilized tissues of plant and animal origin. The concentrations of the unpaired electrons for the various objects were $10^{-6}$–$10^{-8}$ mole/g dry weight. Initiation of enzymatic reactions raised the content of free radicals and heat denaturation completely eliminated the effect.

Similar e.s.r. signals but without a hyperfine structure have been detected by one of the authors [2] in lyophilized tissues of animal origin. The width was of the order of 2–5 gauss and the $g$ factor scarcely differed from that for the free electron. The expenditure of the actual oxidizing substrates before freezing and drying of the preparations caused the e.s.r. signals to vanish and the addition of substrate restored the signals.

The position and width of the line did not vary with the nature of the substrate. In addition, it was noted that the intensity of the e.s.r. line in tissues stored in air gradually declined.

As a result of experiments described in a previous paper [3] the assumption was made that the e.s.r. signal observed is due to unpaired electrons delocalized in the protein structures and of impure origin.

Miiagawa, Gordy, Watabe and Wilbur [4] found that in lyophilized tissues of rat liver preparations no e.s.r. signal or only a very weak one was observed *in vacuo*. When oxygen or air was passed the intensity of the signal rose considerably. These workers put forward the idea that the e.s.r. signal may be produced either by peroxide radicals formed on passage of oxygen or by trapping of oxygen by active groups which are themselves not radicals. The last conclusion is most debatable. In fact, all dry protein preparations adsorb oxygen, but as is known, do not give e.s.r. signals. In addition, no one has succeeded as yet in demonstrating that adsorption of oxygen leads to an increase in the intensity of the e.s.r. signal. On the contrary, it is known, that adsorption of oxygen leads to decrease in the intensity of the e.s.r. signal of certain substances [5].

The assumption that the e.s.r. signal obtained is due to the formation of peroxide radicals is also doubtful. The asymmetric form of the signal is characteristic not only of the peroxide but also any radical in which the unpaired electron is subjected to the action of anisotropic electric fields. The width of the lines of genuine peroxide radicals described in the literature is considerably greater than the width of the signals observed by Gordy *et al.* and by us [6].

It should also be noted that in the paper [4] there is no quantitative information. Moreover, the published work [2, 3] carried out in our laboratory was performed on an e.s.r. apparatus of quite low sensitivity and the earlier conclusions may be open to doubt since the work had to be carried out within the limits of sensitivity of the apparatus.

For these reasons we have studied the effect of oxygen on the e.s.r. spectra of lyophilized tissues of animal origin.

* Biofizika **5**: No. 2, 235–238, 1960.

The e.s.r. spectra were charted on a spectrometer designed by A. G. Semenov with a high frequency magnetic modulation [7]. The sensitivity of the arrangement was $\sim 10^{-11}$ moles. As a standard concentration diphenylpicrylhydrazyl was used with $\sim 1{\cdot}5 \times 10^{15}$ unpaired electrons in the specimen.

The concentration was estimated by two methods: by count of the total intensity and by the amplitude ratio, assuming invariable width. Both methods gave concordant results. The specimens after lyophilization were freed from air in a high vacuum apparatus ($7–9 \times 10^{-6}$ mm Hg). The test objects chosen were lyophilized liver and spleen of male rats. Immediately after killing the animal the tissues were extracted, minced and placed in tubes for lyophilization. This was carried out in two ways: (1) with preliminary freezing of the minced tissues, in milder conditions than in the paper quoted [4] and (2) without freezing. After freezing the tubes containing the minced tissues were placed in a Dewar vessel with liquid nitrogen and the lyophilization begun.

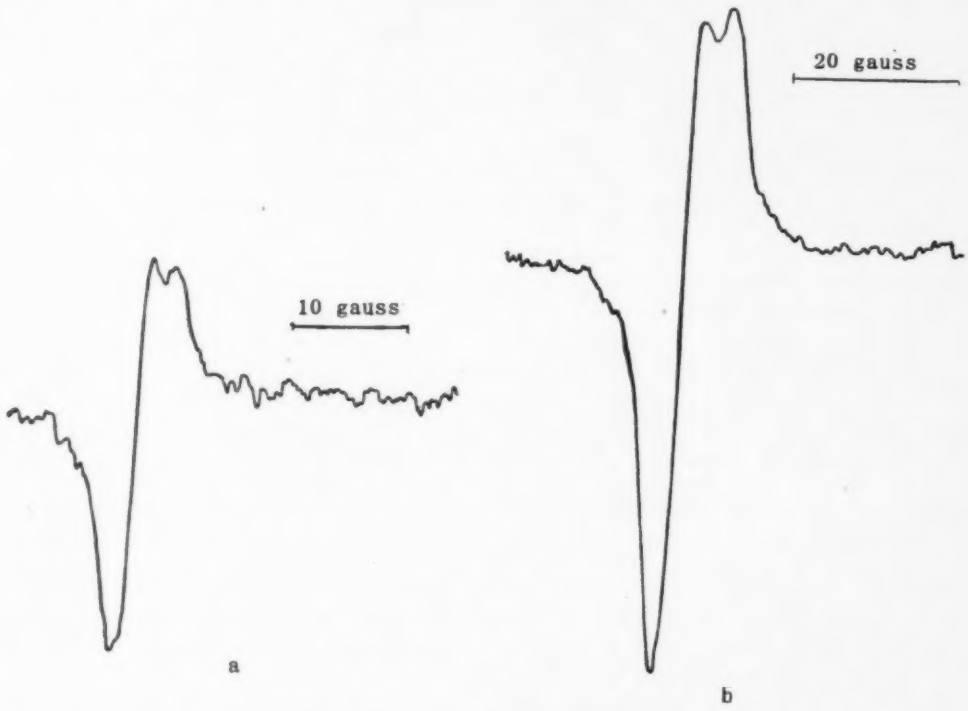


Fig. 1*a*. e.s.r. spectrum of lyophilized preparation of rat liver.
Fig. 1*b*. e.s.r. spectrum of lyophilized preparation of rat spleen. Spectra recorded in form of first derivatives of adsorption lines.

In all the specimens investigated, e.s.r. signals were obtained with the characteristic assymetrical form (see figure). As a rule, the signals from the spleen preparations were 2–3 times more intense. The width between the points of the maximum slope of the signal was 4–5 gauss. The main results of the experiment are given in Tables 1 and 2.

The concentrations of unpaired electrons for the spleen, measurements made immediately following lyophilization, were about $3–5 \times 10^{17}$ per gram dry weight. For the liver the figures were 2–3 times less.

As the tables show, in the case of lyophilization without preliminary freezing keeping the specimens in a vacuum of the order of $10^{-6}$–$10^{-5}$ mm Hg did not lead to any appreciable reduction in the signal. In a number of cases, a 2–3 fold decrease in the intensity of the signal was noted on storing the specimens in air for several days. No change in the shape of the signal was observed in these cases.

With lyophilization of the specimens after preliminary freezing, evacuation, as a rule, led to a reduction in the signal by about one half.

With sufficiently long storage in air there was a tendency for the intensity of the signal to fall.

We did not observe any disappearance of the signal as a result of vacuum formation.

It may be assumed that on lyophilization in more severe conditions, as in the case of Miiagawa *et al.*, the effect may be considerable, which with insufficient sensitivity of the apparatus might lead to an erroneous conclusion on the disappearance of the signal.

TABLE 1. RELATIVE INTENSITY OF E.S.R. SIGNAL OF TISSUES OBTAINED AFTER LYOPHILIZATION WITH PRELIMINARY FREEZING

| Sample | Relative intensity of signal | |
|---|---|---|
| | Liver | Spleen |
| First series | | |
| Tissue before evacuation | 1·0 | 1·0 |
| Tissue after evacuation (1 hr 30 min) at $8\times10^{-6}$ mm Hg | 0·88 | 0·58 |
| Second series | | |
| Tissue before evacuation | 1·0 | 1·0 |
| Tissue after evacuation (3 hr 30 min) at $8\times10^{-6}$ mm Hg | 0·55 | 0·51 |
| Tissue kept in air 2–3 min after evacuation | 0·60 | 0·50 |
| Tissue kept in air 1 hr 50 min after evacuation | 0·51 | 0·63 |
| Tissue kept in air 12 days after evacuation | 0·49 | 0·33 |

TABLE 2. RELATIVE INTENSITY OF E.S.R. SIGNAL OF TISSUES OBTAINED AFTER LYOPHILIZATION WITHOUT PRELIMINARY FREEZING

| Sample | Relative intensity of signal | |
|---|---|---|
| | Liver | Spleen |
| First series | | |
| Tissue before evacuation | 1·00 | 1·00 |
| Tissue after evacuation (1 hr 30 min at $8\times10^{-6}$ mm Hg) | 0·70 | 0·90 |
| Tissue kept in air 1 day after evacuation | 0·44 | 0·49 |
| Tissue before second evacuation | 0·37 | 0·75 |
| Tissue after second evacuation (5 hr 15 min at $7\times10^{-6}$ mm Hg) | 0·72 | 1·25 |
| Tissue after second evacuation (repeat) | 0·64 | 1·28 |
| Tissue kept in air 60 min after evacuation | 0·64 | 1·23 |
| Tissue kept in air 48 hr after evacuation | 0·68 | 0·95 |
| Second series | | |
| Tissue before evacuation | 1·00 | 1·00 |
| Tissue after evacuation (2 hr 20 min at $9\times10^{-6}$ mm Hg) | 0·94 | 1·35 |
| Tissue kept in air 15 min after evacuation | 0·82 | 0·94 |
| Tissue kept in air 20 hr after evacuation | 0·71 | 0·53 |
| Tissue kept in air 7 days after evacuation | 0·57 | 0.51 |

It is well known [8] that as a result of rapid freezing of high polymer preparations, cryolytic rupture of the bonds may occur with free radical formation. These free radicals, which in themselves as a rule give wide e.s.r. lines (tens and hundreds of gauss), may undergo oxidation by atmospheric oxygen. It may be assumed (though this needs checking) that in these processes the free valencies formed as a result of cryolysis migrate into the conduction band of the undestroyed part of the protein polymer and thus appear in the form of the signal of usual shape. In any case, it may be supposed from the data given here that the anomalous effect of oxygen on the e.s.r. signals of lyophilized tissues observed in paper [4] is an artefact caused by the irregular conditions of lyophilization. The e.s.r. spectra observed for mild conditions of lyophilization in crude lyophilized tissues and enzymatic preparations are not associated with adsorption of oxygen.

We take this opportunity to express our sincere thanks to A. E. Kalmanson for his assistance in carrying out some of the experiments.

*Translated by* A. Crozy

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* There is a translation into English of this work.

# SOME OBSERVATIONS ON THE BIOPHYSICAL THEORY OF MEMORY*

V. Iu. Urbakh

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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In the paper by Foerster [1] an attempt was made to construct a quantitative — phenomenological and physical — theory of memory. This theory is based on the fact that any observed phenomenon leaves an impression which can be resolved into a multiplicity of elementary impressions. Each elementary impression is fixed in the apparatus of memory in a definite carrier (it is assumed that a protein molecule may serve as such a memory carrier) and "charges" it. The charged state of the memory

* Biofizika **5:** No. 2, 238–239, 1960.

carrier is separated from the uncharged state by a low energy barrier and it is therefore metastable so that with passage of time "discharge" of these carriers comes about. Since such discharge is a probability process, information in the memory falls off exponentially.* Applying the results of the experiments of Ebbinghaus [2] on the forgetting of learned but meaningless syllables, Foerster found for the average time of forgetting that the value $\tau = 0{\cdot}412$ days $= 3{\cdot}55 \times 10^4$ sec.

As the physical mechanisms of discharge of the memory carriers (transition of the molecule from a higher energy level to a lower one, separated by an energy barrier) Foerster postulates a tunnel effect and later writes: "Quantum mechanics gives the correlation between $\varepsilon$ (the height of the barrier) and the lifetime of the corresponding state:

$$\tau = \tau_0 e^{\varepsilon k/T} \tag{a}$$

In this formula $\tau_0$ represents a constant of about $10^{-14}$ sec.

From this value and from the experimental magnitude of $\tau$ Foerster obtained $\varepsilon \approx 1{\cdot}4$ eV.

The arguments given above and other physical arguments put forward by Foerster are in conflict with the generally known concepts of quantum mechanics. In particular, formula (a) bears no relation to the tunnel effect; in addition, the origin and meaning of the constant $\tau_0$ are incomprehensible.

An attempt is made below to interpret the discharge of memory carriers as a process analagous to heat denaturation of protein molecules in which the molecule passes from a level with high free energy to a lower free energy level (overcoming the activation barrier) under the influence of heat.

This process is irreversible in the sense that heating and cooling does not cause mutual reverse transitions; the higher complexity of the structure lost under the action of heat, cannot be recovered by subsequent cooling, but only by the action of specific agents.

From this point of view the specificity of protein memory molecules lies in the fact that both states must be considered native, since in each of these states the molecule performs a definite biological function — memorizing and forgetting.

As is known from the theory of absolute velocities of reactions [3] the mean time of the reaction (the reciprocal of the reaction velocity constant) is equal to

$$\tau = \frac{h}{kT} e^{\Delta\mu^{\neq}/kT} \tag{b}$$

where $h$ and $k$ are the Planck and Boltzmann constants, and $\Delta\mu^{\neq}$ the augmentation in the chemical potential of the molecule on activation (the height of the activation barrier). As we can see here there are no arbitrary constants. We also note that the activation barrier is not simply energetic; the chemical potential also incorporates an entropy component (of reciprocal sign which facilitates passage through the barrier). As for the tunnel effect, as is pointed out in [3], its role in the processes of passage through the activation barrier is insignificant and may be completely disregarded.

For body temperature $\approx 37$°C (310°K) we have $h/kT = 1{\cdot}55 \times 10^{-13}$ sec so that we get $\Delta\mu^{\neq} = 1{\cdot}07$ eV (per molecule) or the free energy of activation $\Delta F^{\neq} = 24{\cdot}5$ kcal/mole.

The experimentally-found values for the free energy of 20 per cent denaturation of different proteins are given in [4]. If we convert them to a temperature of 310°K and then average, we get $\Delta F^{\neq}_{mean} = 27{\cdot}2 \pm 2{\cdot}9$ kcal/mole; in general, the values of $\Delta F^{\neq}$ for these proteins vary from 20·2 to 34·3 kcal/mole.

Thus, the above value for $\Delta F^{\neq}$ for discharge of memory carriers comes within this interval.

*Translated by* A. Crozy

* Here we shall not consider at all the process of duplication of excitation of elementary memory carriers ensuring, according to the phenomenological theory of Foerster, the prolonged retention in the memory of part of the information introduced into it.

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# PROBLEM OF THE PROTECTIVE ACTION OF THIOUREA*


IU. A. KRIGER and I. M. PARKHOMENKO

Faculty of Fundamental Biology of the Lomonosov State University, Moscow


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VOL 5 196

THE relationship between the protective action of a given substance and its concentration is of great practical importance for radiobiology since the vast majority of antiradiation substances are highly toxic. However, despite the practical interest of this problem it has received little attention.

Some information on the relation between the effect of protective substances and their concentration is provided in papers by Flemming [1] who investigated the protective action of cysteine, ascorbic acid and gluthathione on the swelling of erythrocytes induced by X-rays. He established that the maximum protective action of these substances was displayed at a concentration of $3\times10^{-3}$ M. At a concentration of $3\times10^{-4}$ M they no longer exerted such an appreciable effect and at even lower concentrations were quite ineffective. On the other hand, use of cysteine at higher concentrations ($3\times10^{-2}$ M) showed it capable of intensifying the swelling of erythrocytes exposed to X-rays and preceded haemolysis.

In the present work our aim was to study the dependence of the protective action of thiourea on its concentration. According to data in the literature [2, 3] thiourea exerts a protective action only at high, near toxic concentrations.

## MATERIAL AND METHODS

The test object in our work was rat erythrocytes first separated from citrated plasma by centrifugation and then washed three times with saline with repeat centrifugation for 10 min at 2000 r.p.m. To 1 ml of the dense suspension was added 99 ml saline, it was carefully shaken and the 1% red cell suspension thus obtained exposed to radiation with a GUT-400 unit for 100 min at a dose rate of 400 r/min (total dose of 40 kr). After irradiation the suspension of erythrocytes was kept for a day in a refrigerator, then centrifuged. The percentage haemolysis was determined by colorimetry of the supernatant using an FEK-52 photoelectric colorimeter. Preliminary calibration was made with the supernatant obtained on centrifugation of 0·5 per cent red cell suspension in distilled water and that corresponding to 50 per cent haemolysis (the supernatant thus obtained was diluted 2·5 and 10 times).

* Biofizika **5**: No. 2, 239–242, 1960.

In the test suspensions of erythrocytes we used solutions of thiourea in saline and having the concentrations: $1{\cdot}25\times10^{-3}$; $2{\cdot}5\times10^{-3}$; $3{\cdot}75\times10^{-3}$; $5{\cdot}0\times10^{-3}$; $1{\cdot}25\times10^{-2}$ and $2{\cdot}5\times10^{-2}$ moles/litre. The supernatant obtained after irradiation of 1% red cell suspension in saline served as control throughout.

## RESULTS

The experimental results are set out in the form of a diagram (Fig. 1). It will be seen that thiourea in large concentrations ($2{\cdot}5\times10^{-2}$ and $1{\cdot}25\times10^{-2}$ M) appreciably protected the erythrocytes from the action of γ-rays. The solutions of thiourea with concentrations of $5{\cdot}0\times10^{-3}$ and $3{\cdot}75\times10^{-3}$ M exerted weak protective action and at still lower concentrations of thiourea ($2{\cdot}5\times10^{-3}$; $1{\cdot}25\times10^{-3}$) haemolysis was greater than in the solution not containing thiourea.

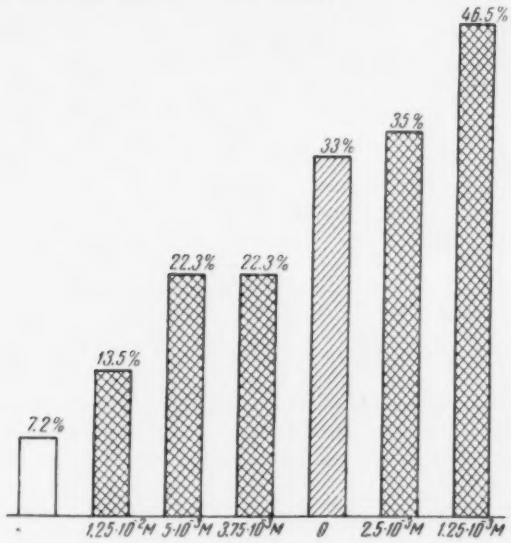


FIG. 1. Dependence of the degree of haemolysis due to the action of γ-rays on the concentration of thiourea. (*a*) unirradiated control; (*b*) irradiation in absence of thiourea; (*c*) irradiation in presence of thiourea. 1, $1{\cdot}25\times10^{-2}$ M; 2, $5\times10^{-3}$ M; 3, $3{\cdot}75\times10^{-3}$ M; 4, $2{\cdot}5\times10^{-3}$ M; 5, $1{\cdot}25\times10^{-3}$ M.

The most likely explanation of the effect observed by us lies in the pattern of penetration of thiourea into the red cells (the erythrocytes were permeable to it at low concentrations and impermeable at higher ones).

In order to test the validity of our assumption we carried out similar experiments on intact red cells not previously exposed to radiation. As the results of the investigations showed, the solutions of thiourea at low concentrations ($2{\cdot}5\times10^{-3}$ M and less) did in fact exert a weak haemolytic action whereas the solutions containing higher concentrations of thiourea ($1{\cdot}25\times10^{-2}$ M) did not have this effect. The haemolytic action noted by us of low concentrations of thiourea was less marked than in the case of the irradiated erythrocytes, which is by no means surprising considering that radiation, as a rule, raises the sorption level of protoplasm [4].

As a final check on the accuracy of our assumption we studied the effect of various concentrations of thiourea on the course of a different type of haemolysis, namely, photodynamic haemolysis. If it could be demonstrated that the dependence of the protective action of thiourea on its concentration with photodynamic and radiation haemolysis was of identical character, then the assumption made would be beyond doubt.

Photodynamic haemolysis was carried out by irradiating a 0·01% erythrocyte suspension in 0·04% solution of erythrosin prepared in saline. The erythrocytes were added to the samples containing erythrosin solutions to which had first been added thiourea. The concentration of the latter was the same as in the experiments with radiation haemolysis ($1{\cdot}25\times10^{-3}$; $5\times10^{-3}$; $1{\cdot}25\times10^{-2}$; $2{\cdot}5\times10^{-2}$ M). Irradiation was carried out for 10 min placing the vessel containing the erythrocyte suspension at a distance of 25 cm from a 500 W incandescent lamp. To eliminate the possible effect of heat emission from the lamp a flat-parallel sided vessel 8·2 cm thick, filled with 0·5% copper sulphate solution, was used as a filter.

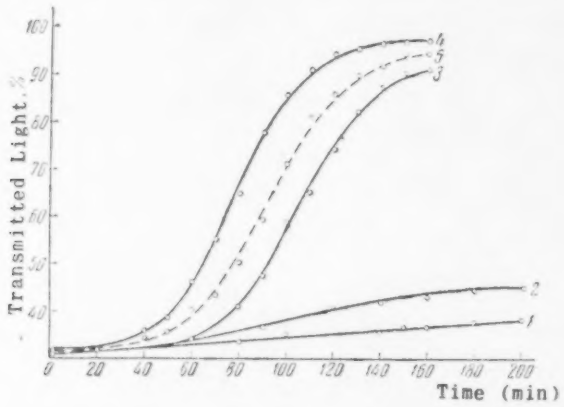


Fig. 2. Dependence of photodynamic haemolysis on concentration of thiourea. Abscissa indicates time (min); Ordinates, transmitted light as percentage. ------ denotes absence of thiourea; ——— presence of thiourea. 1, $2{\cdot}5\times10^{-2}$ M; 2, $1{\cdot}25\times$ $\times10^{-2}$ M; 3, $5\times10^{-3}$ M; 4, $1{\cdot}25\times10^{-3}$ M.

The course of haemolysis was recorded from the change in the light transmitting properties of the erythrocyte suspension by the method of nephelometry on an FEK-52 photoelectric colorimeter. The measurements were made every 10 min for 3–4 hr.

The results of the measurements are given in Fig. 2 where the abscissa is the time in min and the ordinate the transmitted light as a percentage of that of the erythrosin solution used but not containing erythrocytes. It follows from the graph that on photodynamic haemolysis, as with radiation damage to the erythrocytes, thiourea exerts a protective action only at relatively high concentrations ($1{\cdot}25\times10^{-2}$; $2{\cdot}5\times10^{-2}$ M). At lower concentrations $5\times10^{-3}$ M, thiourea exerted only a very weak protective action and at the concentration of $1{\cdot}25\times10^{-3}$ M not only failed to protect the erythrocytes but, on the contrary, produced additional haemolysis.

Thus, in the case of both photodynamic and radiation haemolysis, similar quantitative relations were observed between the concentration of thiourea in the solution and its protective action.

## DISCUSSION OF RESULTS

From the uniform nature of the dependence of the protective action of thiourea on its concentration in both photodynamic and radiation haemolysis it may be assumed that the regularities observed have the same cause, which, as already pointed out, may be the ability of thiourea to penetrate the erythrocytes at low concentrations and its inability to do so at higher concentrations in solution. Similar relations, as is known, have been obtained by Troshin [5] for urea. The character of the distribution of the latter between the muscle fibres and the washings was wholly dependent on its concentration in the external medium. At low concentrations urea penetrated the muscle so strongly

that its concentration in the muscle became higher than in the surrounding solution. At higher concentrations penetration of the urea in the muscles, on the other hand, was insignificant so the content of urea in the fibres was correspondingly lower than in the washings. Apparently, the distribution of thiourea between the irradiated erythrocytes and the solution washing them is of the same character. It is very likely that the phenomena observed are due to the ability of urea and its derivatives to bind with proteins, and thereby to the accumulation of these compounds within the protoplasm.

Recently, there have appeared in the literature data indicating the possibility of formation of more or less firm compounds between proteins and urea and thiourea of the "inclusion compound type" [6]. Such compounds are probably formed only at low concentrations of urea and its derivatives and on the contrary are absent at higher concentrations of these substances.

## SUMMARY

(1) The degree of radiation and photodynamic haemolysis falls at relatively high concentrations of thiourea in the washing solution ($1{\cdot}25\times10^{-2}$ M and higher). At lower concentrations ($5\times10^{-3}$ M) thiourea exerts very weak protective action and with a further decrease in concentration ($2{\cdot}5\times10^{-3}$; $1{\cdot}25\times10^{-3}$ M) causes intensification of haemolysis.

(2) The experimentally found dependence of the protective action of thiourea on its concentration is apparently to be explained by the ability of thiourea to penetrate the erythrocytes at relatively low concentrations and the inability to do so at relatively high concentrations. It is assumed that the phenomena observed are based on the ability of thiourea to form "inclusion compounds" with proteins of the protoplasm.

*Translated by* A. CROZY

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# ULTRASONIC IMAGES OF THE ORGANS AND TISSUES OF THE ANIMAL ORGANISM*

I. Ye. EL'PINER, I. M. DRIZE and I. M. FAIKIN

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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IN a previous communication [1] material was presented showing that study of the phenomena of reflexion of ultrasonic waves in biological media can give valuable information on their macrostructure.

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Still earlier Wild and Reid [2, 3] established that the organs and tissues abound in ultrasonic "contrast" media causing the appearance of a large number of reflected signals (echos) of different amplitudes.

This was a method for ultrasonic biolocation which helped to reveal fundamental differences not only between different tissues, but also between normal tissue macrostructures and pathological neoplasms. It should be noted that further developments in ultrasonic techniques have made it possible to obtain both two dimensional and three dimensional images of the test tissues [4, 5].

In the present paper we give the images of organs and tissues obtained by the method of two dimensional ultrasonic biolocation (method of ultrasonic scanning). With this method the reflected ultrasonic pulse is presented in the form of a small fluorescent spot or point on the synchroscope. The greater the intensity of the reflected pulse, the brighter the fluorescence of the corresponding point on the screen. If the ultrasonic pulse is reflected from several surfaces arranged consecutively, then on the screen there appears a set of fluorescent points drawn out into a straight line. By giving the suceeding pulse a somewhat different direction, we obtain on the screen a new line of fluorescent points, contiguous with the preceding one. On rapid movement of the emitter, the ultrasonic beam repeatedly intersects the test object producing on the screen a continuous image of the cross section of the object [5].

A block diagram of the apparatus is given in Fig. 1. The frequency of the ultrasonic vibrations is 4 Mc/s, pulse frequency 1000 c/s and duration of pulse 1·2 μsec. The ultrasonic wave source is a piezo-quartz plate of 15 mm diameter; this also serves as the receiver of the reflected ultrasonic signals.

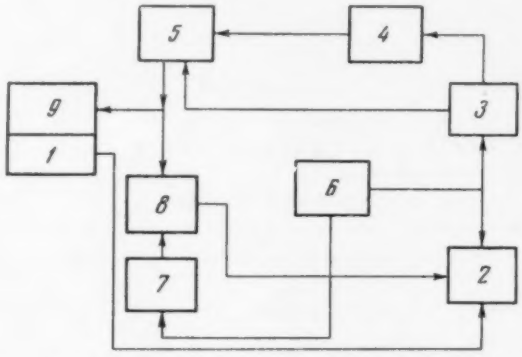


Fig. 1. Block diagram of apparatus. *1*, position data unit; *2*, indicator; *3*, modulator; *4*, master oscillator; *5*, power amplifier; *6*, synchronous shutter; *7*, synchronator; *8*, receiver; *9*, scanning sonic head.

The head of the device with the piezo-quartz plate is immersed in a bath filled with distilled water. The emitting plate, by means of mechanical arrangement, is moved in the bath in a horizontal direction at a definite depth. The test object is immersed in the water at a fixed distance from the head of the apparatus.

In our description we shall confine ourselves to a discussion of the ultrasonic images which make it possible to judge the resolving power and the prospects for the method of investigation described.

Fig. 2 (a) gives the ultrasonic image of the cross-section of an isolated specimen of the back muscles of a pig; the individual muscle bundles separated from one another by the connective tissue septa are visible here. In Fig. 2 (b) the image of the same muscle tissue but with the insertion in a vertical direction in the left half of the muscle layer of a bone plate 0·1 mm thick is given. As this figure shows, the ultrasonic energy from this plate is completely reflected accompanied by the appearance of the so-called ultrasonic shadow. A similar shadow was obtained on replacing the bone by a glass plate, wood, etc.

In Fig. 2 (c) we give the ultrasonic shadow developed as a result of reflexion of the waves from a renal calculus introduced into the test muscle tissue (thickness of calculus 2 cm, length 3 cm). The same figure shows that the renal calculus introduced into the muscle layer caused the curvature of

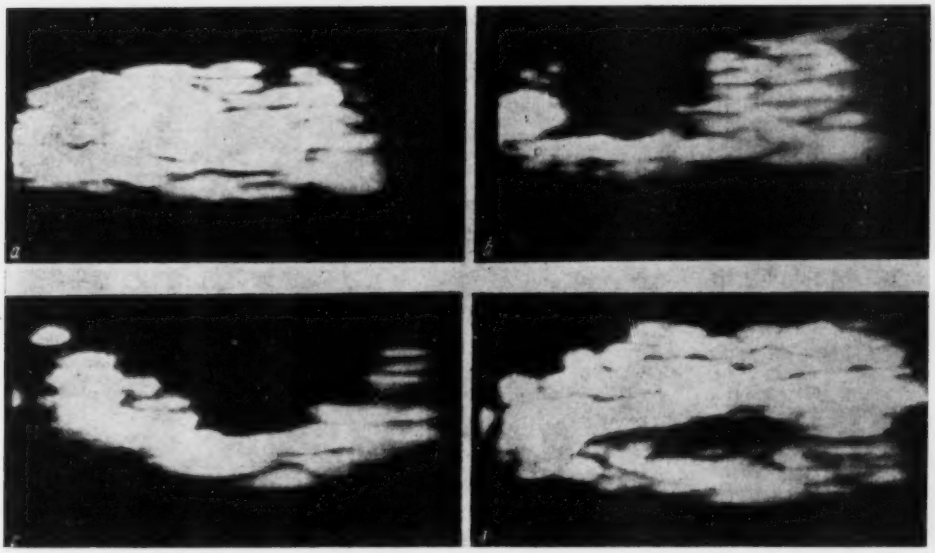

FIG. 2. Ultrasonic image of certain biological tissues (explanation in text).

the anterior margin of the muscle. On removal of the calculus a water-filled recess remained in the muscle tissue as seen in Fig. 2 (d).

The isolated renal calculus not protected by the muscle tissue did not completely reflect the incident ultrasonic energy. The latter penetrated deep into the calculus, making it possible to obtain the image

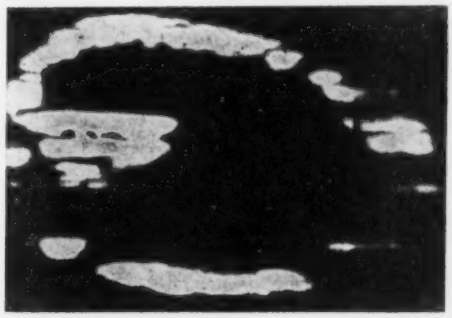

FIG. 3. Ultrasonic image of stretched rabbit stomach containing a polymethyl methacrylate plate.

of its cross-section and to establish the structure of the calculus. This apparatus also enabled us to obtain an image of the cross-section of the rabbit stomach and to reveal the presence in it of foreign bodies; a piece of polymethyl methacrylate, pencil, nail, piece of liver tissue, etc. (Fig. 3). Through

the facial bones of the rabbit cranium it was found possible to obtain an image of the antrum of Highmore filled with water. It was also easy to detect in this antrum the presence of various objects earlier inserted.

Of extreme interest are the images obtained of the horizontal section of the eye in which the crystalline lens, vitreous body and the membranes of this organ are visible.

The investigations show that ultrasonic visualization may in fact be very promising for the study of the macrostructures of normal and pathological tissues of the intact organisms and the scientific and practical importance of such investigations is obvious.

*Translated by* A. CROZY

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VOL
5
196

# BIOPHYSICS IN PRACTICE

# DATA ON THE RADIOSENSITIVITY OF AGRICULTURAL CROPS*

S. A. VALEVA

IN the Laboratory of Radiation Genetics of the Institute of Biophysics of the U.S.S.R. Academy of Sciences, a composite table has been drawn up of the radiosensitivity of seeds of different agricultural crops. The compilation includes both data in the literature and as yet unpublished material obtained by the staff of the Laboratory and also in other scientific institutions.

The purpose of the present list is to facilitate the choice of radiation doses — chiefly for plant selection work.

The criterion of sensitivity adopted was the so-called critical dose, a term first introduced by Gustafsson, i.e. the dose at which appreciable inhibition of plant development is observed, but with a sufficient number of plants at the end of vegetation capable of giving seeds (30–20 per cent). The doses are given in roentgens. For certain plants, the critical dose has not been precisely defined and the information given is only approximate. Thus, the signs $>$ and $<$ denote that the critical dose is greater or less than that indicated but close to it; $\geqslant$ denotes that the critical dose is much greater than that indicated.

* Biofizika **5**: No. 2, 244–248, 1960.

SENSITIVITY OF CULTIVATED PLANTS TO γ AND X-RADIATIONS

| Crop, variety | Exposed to radiation | Critical dose in roentgens | Author |
|---|---|---|---|
| 1 | 2 | 3 | 4 |
| Klinelimus | Dried seeds | < 5000 | Sorokina |
| Timothy-grass | ,, ,, | <10,000 | |
| Regneriia | ,, ,, | < 5000 | |
| Maize | | | |
| Dniepropetrovsk | ,, ,, | 10,000 | Preobrazhenskaia |
| Krasnodar hybrid | ,, ,, | 10,000 | Valeva |
| Kuban Liming | ,, ,, | 10,000 | |
| Vir 42 | ,, ,, | 10,000 | |
| Voronezh 76 | ,, ,, | 10,000 | |
| Moscow region | ,, ,, | 10,000 | |
| Nemchinov | ,, ,, | 12,000–15,000 | |
| Hybrids with Nemchinov | ,, ,, | 12,000–15,000 | |
| Leninskiye Gorki No. 1 | ,, ,, | 15,000 | Orav |
| Rye | ,, ,, | 10,000–15,000 | Gustafsson |
| Viatka | ,, ,, | 20,000 | Valeva |
| Meadow grass | ,, ,, | 15,000–20,000 | Gustafsson |
| Canary grass | ,, ,, | 16,000 | Khertssh |
| ,, ,, | Germinating seeds | 8000 | |
| Two-rowed barley | Dried seeds | 20,000–25,000 | Preobrazhenskaia |
| ,, ,, ,, | Pollen in period of maturation | 2000–2500 | Lutkov |
| | Germinating seeds | 12,000 | |
| Oats | Dried seeds | 25,000–30,000 | Gustafsson |
| Pobyeda | ,, ,, | 25,000 | Preobrazhenskaia |
| Nemchinov | ,, ,, | 30,000 | Sorokina |
| Millet | | | |
| Krasnoufa | ,, ,, | >25,000 | Preobrazhenskaia |
| Einkorn | ,, ,, | <15,000 | Gustafsson |
| Emmer | ,, ,, | >15,000 | |
| Durum wheat | | | |
| Akmolinka | ,, ,, | 15,000 | Sorokina |
| Wheat | | | |
| Mil'turum | ,, ,, | 5000 | Preobrazhenskaia |
| Northern | ,, ,, | ⩾5000 | |
| Branchy | ,, ,, | >5000 | |
| Lutesence | ,, ,, | ~15,000 | Orav |
| Moscow | ,, ,, | <20,000 | Valeva |
| Diamant | ,, ,, | ~15,000 | Priilinn |
| Kauka | ,, ,, | 15,000 | |
| Motsinava | ,, ,, | 15,000 | Grdzelishvili |
| Tsikho 1 | ,, ,, | 15,000 | |
| Kakhi 8 | ,, ,, | 15,000 | |
| Erythrospermum 143 | ,, ,, | 15,000 | |
| Suidin | ,, ,, | 20,000 | Kobylianskaia |
| Hybrid 1429 | ,, ,, | 20,000 | |

(Contd.)

| Crop, variety | Exposed to radiation | Critical dose in roentgens | Author |
|---|---|---|---|
| 1 | 2 | 3 | 4 |
| Hybrid 186 | Dried seeds | >20,000 | Valeva |
| Hybrid 1 | ,, ,, | >20,000 | |
| Hybrid 99 | ,, ,, | 20,000 | |
| Hybrid 557 | ,, ,, | 20,000 | |
| Ul'ianovka | ,, ,, | 20,000 | |
| Meadow foxtail grass | ,, ,, | 26,000 | |
| Rye grass | ,, ,, | 40,000 | Sorokina |
| Sorghum | | | |
| Kafrskoye | ,, ,, | >30,000 | Nikolayeva |
| Mailo | ,, ,, | >30,000 | |
| Feterita | ,, ,, | >30,000 | |
| Dzhugari | ,, ,, | >30,000 | |
| Pea | ,, ,, | 5000—15,000 | Gustafsson |
| ,, | Buds, barely coloured | 2000 | Lutkov |
| Zhegalovskii | Dried seeds | 5000 | Turkov |
| Victoria Shtrube | ,, ,, | 5000 | Preobrazhenskaia |
| Sugar | ,, ,, | 5000 | |
| Victoria pink | ,, ,, | >5000 | |
| Shtambov | ,, ,, | >5000 | |
| Kapital | ,, ,, | 15,000 | |
| Moscow | ,, ,, | <10,000 | Valeva, Turkov |
| Nemchinov | ,, ,, | 10,000 | |
| Rainiai | ,, ,, | 15,000 | |
| Peliushka | | | |
| Small seed | ,, ,, | ~50,000 | |
| Broad beans | ,, ,, | 7000 | |
| Haricot | | | |
| VIR variety | ,, ,, | <8000 (near Leningrad) | Makasheva |
| ,, ,, | ,, ,, | >8000 (in the Ukraine) | |
| Haricot | ,, ,, | | |
| Robust | ,, ,, | <10,000 | Valeva |
| Tyuchadze | ,, ,, | <10,000 | |
| Chokhatau | ,, ,, | 10,000 | |
| 1019 | ,, ,, | 10,000 | |
| 1020 | ,, ,, | 10,000 | |
| Izumrud | ,, ,, | 10,000 | |
| Saint Andrei | ,, ,, | >10,000 | |
| Krasnyi rubchik | ,, ,, | >10.000 | |
| Latvia, dried seed | ,, ,, | >10,000 | |
| White Moscow | ,, ,, | 25,000 | Preobrazhenskaia |
| Northern | ,, ,, | 25,000 | |
| Phaseolus mung | | | |
| 4730 | ,, ,, | 30,000 | Turkov |
| Soya | ,, ,, | 7000 | |

(Contd.)

| Crop, variety | Exposed to radiation | Critical dose in roentgens | Author |
|---|---|---|---|
| 1 | 2 | 3 | 4 |
| Kuban 276 | Dried seeds | ~12,000 | Mitiukevich |
| Kuban 4958 | ,, ,, | 20,000 | Mitiukevich |
| Hybrid 29 | ,, ,, | 20,000 | Soldatova |
| Vigna | | | |
| 292 | ,, ,, | 12,000 | Turkov |
| White vetch | | | |
| Steppe | ,, ,, | 20,000 | Preobrazhenskaia |
| Lentil | | | |
| 1900 | ,, ,, | 12,000 | Turkov |
| Dnepropetrovsk | ,, ,, | 20,000 | Preobrazhenskaia |
| Yellow lupin (food) | ,, ,, | 20,000 | Priilinn |
| Annual lupin (alkaloid) | ,, ,, | 60,000 | Valeva |
| Vetch | | | Valeva |
| 31 | ,, ,, | 10,000 | Valeva |
| L'gov | ,, ,, | 25,000 | Preobrazhenskaia |
| Peanut | ,, ,, | 25,000 | Preobrazhenskaia |
| ,, | ,, ,, | 20,000 | Gregori |
| Chick-pea | | | |
| 1168 | ,, ,, | 25,000 | Valeva |
| Bird's foot trefoil | ,, ,, | 80,000 | Valeva |
| Lucerne | ,, ,, | 100,000 | Preobrazhenskaia |
| Clover | ,, ,, | 100,000 | Preobrazhenskaia |
| Sweet clover | ,, ,, | 100,000 | Preobrazhenskaia |
| Onion | | | |
| Strigunov | ,, ,, | <10,000 | Turkov |
| Lettuce | | | Turkov |
| Bettner | ,, ,, | 10,000 | Turkov |
| Sugar beet | ,, ,, | 10,000 | Berezina |
| Cotton | ,, ,, | 50,000 | Giul'akhimedov |
| ,, | Pollen | 2000 | Osborne |
| Perilla | Dried seeds | 15,000–20,000 | Preobrazhenskaia |
| Tomatoes | | | |
| Pushkin | ,, ,, | 20,000 | Turkov |
| Urozhai | ,, ,, | 20,000 | Turkov |
| Potato | Tubers | 5000 | Rubin |
| ,, | ,, | 5000–10,000 | Niubom |
| Sunflower | Dried seeds | 7000 | Gustafsson |
| Melon | | | |
| Kolkhoznitsa | ,, ,, | >20,000 | Soldatova |
| Spinach | | | |
| Zhiriolist | ,, ,, | 25,000 | Preobrazhenskaia |
| Fennel | ,, ,, | 30,000 | Preobrazhenskaia |
| Buckwheat | | | |
| Bol'shevik 2k | ,, ,, | 20,000 | Sakharov *et al.* |
| ,, 4k | ,, ,, | 40,000 | Sakharov *et al.* |

(Contd.)

| Crop, variety | Exposed to radiation | Critical dose in roentgens | Author |
|---|---|---|---|
| 1 | 2 | 3 | 4 |
| Hemp | | | |
| Krasnodar | Dried seeds | 30,000 | Markovich |
| ,, | Germinating seeds | 8000–10,000 | Lutkov |
| Cucumber | | | |
| Moscow region | Dried seeds | 50,000 | Preobrazhenskaia |
| Viaznikov | ,, ,, | ~50,000 | Turkov |
| *Nicotiana rustica* | ,, ,, | >50,000 | Preobrazhenskaia |
| ,, | Pollen | 14,000–17,000 | Lutkov |
| Castor plant | | | |
| Novo-Kuban | Dried seeds | >50,000 | |
| Sesame | ,, ,, | >50,000 | Preobrazhenskaia |
| *Lactarius deliciosus* | ,, ,, | 50,000 | |
| Turnip | ,, ,, | 35,000 | Gustafsson |
| Cauliflower | ,, ,, | 80,000 | Turkov |
| Cabbage | ,, ,, | >100,000 | Gustafsson |
| Late Moscow | ,, ,, | >80,000 | |
| Carrot | ,, ,, | 80,000 | Turkov |
| Rice | ,, ,, | >75,000 | Bora |
| Indian mustard | ,, ,, | 100,000 | Turkov |
| White mustard | ,, ,, | >100,000 | Gustafsson |
| Rape | | | |
| Nosov | ,, ,, | 100,000 | |
| Radish | | | |
| Winter | ,, ,, | >100,000 | |
| Chinese bell flower | | | Preobrazhenskaia |
| 085 | ,, ,, | 100,000 | |
| Liallemansia | ,, ,, | 100,000 | |
| Flax | | | |
| Climbing | ,, ,, | >100,000 | Turkov |
| ,, | ,, ,, | 200,000 | Preobrazhenskaia |
| Radish | | | |
| Rubin | ,, ,, | >100,000 | Turkov |
| Saksa | ,, ,, | 200,000 | Preobrazhenskaia |
| Garlic | Bulb | 100 | Giul'akhmedov |
| Strawberry | Dried seeds | 20,000 | |
| | Runners | 150 | Mochalova |
| Citrus (Lemons, Oranges, Grapefruit, etc.) | Pollen | 8000 | |
| | Dried seeds | ~2000 | Sakharulidze |
| Blackcurrant | Grafts | 3000 | Bauer |
| Kernel fruits | Resting buds | 2500–4000 | |
| Apple trees | ,, ,, | 5000–7500 | Grankhal' |
| Pomegranate | Dried seeds | ~10,000 | |
| Quince | ,, ,, | ~10,000 | Radzhabli |
| Grape | Vines | 7500 | Breider |
| ,, | Dried seeds | ~10,000 | Radzhabli |

(Contd.)

| Crop, variety | Exposed to radiation | Critical dose inroentgens | Author |
|---|---|---|---|
| 1 | 2 | 3 | 4 |
| Wood species | Buds in quiescent stage | 12,000 | Trudova |
| ,, ,, | Buds in period of summer maturation | 1500 | |
| Ordinary pine | Dried seeds | >900 | Gustafsson |
| Fir | ,, ,, | >900 | |
| Alder | ,, ,, | >10,000 | |
| Mulberry tree 2p | ,, ,, | <10.000 | Radzhabli |
| 4p | ,, ,, | 20,000 | |
| Spirea | ,, ,, | 30,000 | Soldatova |
| Tulip | Bulb | 600 | Lusdrekht |
| Cyclamen | ,, | 10,000 | Reikhardt |
| *Phlox Drumondi* Defiance | Dried seeds | <5000 | Priilinn |
| *Petunia hybrida* Comanche | ,, ,, | <5000 | |
| *Delphinium hibridum* | ,, ,, | <5000 | |
| *Callistephus chynensis* Markt | ,, ,, | 5000 | |
| *Chrysanthemum indicum* | ,, ,, | 7000 | Iank |
| *Carthamus tinctorius* | ,, ,, | 7000 | Gustafsson |
| *Lathyrus odoratus* 3999 | ,, ,, | 10,000 | Priilinn |
| *Antirrhinum majus* Scharlach triumph | ,, ,, | <10,000 | |
| *Calendula officinalis* | ,, ,, | <15,000 | |
| *Gladiolus gandavensis* | Bulbs | >15,000 | |
| *Dianthus caryophyllus* | Dried seeds | >15,000 | |
| *Aquillegia hybrida* | ,, ,, | >15,000 | |
| *Papaver somniferum* | ,, ,, | ~15,000 | Gustafsson |
| *Polygonum lapathifolium* | ,, ,, | 16,000 | Utekhin |
| *Mathiola incana* Teicheri Rubin | ,, ,, | 15,000 | Priilinn |
| Dresden | ,, ,, | 25,000 | Shcherbakov |
| *Elscholtia* | ,, ,, | 20,000 | Kolomiyets |
| *Datura tatula* | ,, ,, | 20,000 | Shteiniger |
| *Datura stramonium* | ,, ,, | 20,000 | |
| *Phacelia cetifolia* | ,, ,, | >50,000 | Khvostova |
| *Stellaria media* | ,, ,, | 64,000 | Utekhin |
| *Amaranthus retroflexus* | ,, ,, | ⩾64,000 | |

*Translated by* A. CROZY

VOL.
5
196

# ON THE NINETIETH ANNIVERSARY OF THE BIRTH OF THE GREAT LENIN *

THE twentieth century is a period of swift development in the natural sciences, thorough demolition of old traditions and a time of unexpected discoveries and achievements in science.

Only the great generalizing mind of V. I. Lenin could truly assess the significance of the discoveries of natural science.

The appearance of his immortal work "Materialism and Empirico-criticism" was the source of the subsequent accelerated development of science, the shattering of old ideas and theoretical concepts, which is still continuing today more than 50 years after the publication of V. I. Lenin's book.

The conclusions drawn by V. I. Lenin from the most modern generalizations of sciences are applicable to an analysis of the contemporary state of knowledge to an even greater degree than 50 years ago.

The exceptional importance of the dialectical materialist epistemology has been repeatedly borne out in practice and is confirmed by the whole course of development of science.

A characteristic feature of the modern state of natural science is the ever increasing interrelation of biology, chemistry and physics. The chemical and physical interpretation of a whole range of biological phenomena has ceased to be hypothetical and guesswork, and has become the basis of the work of the experimenter and theoretician. Metabolism lies at the basis of life and all that physics gives to chemistry is also utilized by biology. The special orderliness of biological structures and the vast ramified boundary surfaces peculiar to living matter create unusual catalytic possibilities.

The discoveries of biology require new theoretical generalizations, creative boldness and innovation, qualities which V. I. Lenin called for.

The great ideas of V. I. Lenin are unextinguishable and his teachings on matter and motion are the foundation of contemporary natural science. It is these ideas of Lenin which must guide us in investigating the chemical and physical bases of the special form of motion of matter called life in the epoch of the turning point in biology, when physics and chemistry are proving singularly effective in discovering the secrets of living matter.

*Translated by* A. CROZY

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# CHARACTERISTICS OF AGEING OF A GEL-SOLUTION OF ACTOMYOSIN*

IU. N. POLIANSKII

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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HIGHLY concentrated solutions of actomyosin have long been used in preparing actomyosin threads [1]. In particular, the specific action of ATP on the mechanical properties of actomyosin has been established on such threads [2].

However, so far little study has been made of the structural-mechanical properties of the most concentrated solutions of actomyosin, especially those with a protein concentration greater than 2 per cent and with an ionic strength of more than 0·3, which we shall term gel-solutions. It is not even known how these properties vary on storage of the preparation.

Apparently, the reason for this is failure to devize suitable methods of investigating such objects possessing low fluidity, sharply pronounced abnormal viscosity, thixotropy and elasticity. The application of the conventional method of structural viscometry is hardly justified in this case [3].

There have recently appeared new rheological methods [4, 5] making it possible to study quantitatively the structural-mechanical properties of systems similar to the actomyosin gel-solution.

We sought to apply one such method (in which $P$, the tangential stress, is constant) to elucidate the features of ageing of an actomyosin gel-solution. This method [4] makes it possible to study the ultra-mycelial structure of the test object in the virtually unaffected form. It consists in plotting the curves of development of the shear deformation on application of, and maintenance of, a constant shear stress in a narrow gap between two coaxial cylinders, where a practically uniform field of stresses is set up.

From these curves (curves $\varepsilon = \varepsilon(t)$ for $P = \text{const.}$) we computed the nominal momentary modulus of elasticity (shear) $E_1 = P/\varepsilon_0$, the elastic modulus $E_2 = (P)/\varepsilon - \varepsilon_0$, the equilibrium modulus $E = P/\varepsilon$ and the relaxation viscosity $\eta_1 = P/(\mathrm{d}\varepsilon/\mathrm{d}t)_{\text{residual}}$ ($P$, the tangential stress; $\varepsilon_0$, the relative shear deformation for $t \approx 1{\cdot}5$ sec; $\varepsilon$, the complete relative deformation developing during the experiment; $d_\varepsilon/d_t$ the rate of increase in the residual relative deformation).

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These parameters are invariants [4, 6], i.e. do not vary with the design of the apparatus or, within certain limits, with the shear stress applied. They are determined solely by the features of the structure and the heat motion of the elements of the structural network of the system studied, and also by the interaction between the elements of the structure and the dispersion medium.

The actomyosin gel-solutions investigated by us were prepared as follows. The rabbit muscle substance was extracted for a day in the cold by 3 times its volume of Bailey's solution. The suspension after dilution with the same solution (1:0·8) was filtered. The filtrate was diluted 10 times with cold double-distilled water. A residue formed at pH 6·8 and was centrifuged and adjusted to a final concentration of 0·5 M KCl and 0·03 M $NaHCO_3$ and stored in closed tubes at 0–2°. The protein concentration was determined on the dry weight.

OL.
5
960

TABLE 1

| Measurements | $E_1$ dynes. $cm^{-2}$ | $E_2$ dynes. $cm^{-2}$ | E dynes. $cm^{-2}$ | $\eta_1$ $10^3$ poises |
|---|---|---|---|---|
| I | 67 | 14 | 11 | 4·6 |
| II | 96 | 50 | 32 | 11·3 |
| III | 114 | 89 | 49 | 21·8 |

Note: Thixotropy of 2·4 per cent gel-solution of actomyosin at 8°. Measurements: (I) 30 min after destruction for $G = 150$ $sec^{-1}$ during 1 min; (II) 60 min after measurement I, without preliminary destruction of structure; (III) 90 min after measurement II without preliminary destruction of structure.

During 7 days the preparations remained homogenous (synaeresis was not observed). The ATP-ase activity fell somewhat (at the start $Q_p = 4000$–$3000$, after a week $Q_p = 3000$–$2000$), the pH dropped from 8·1 to 7·8.

The measurements were made by complex elasto-viscometry, after Trapeznikov [5, 7]. Cylinders were used: internal radius 1·4 cm, height, 10·0 cm, external radius, 1·5 cm. The working clearance between the cylinders was 0·1 cm. The internal cylinder had a corrugated lateral surface, and a spherical recess to eliminate slipping. The curves $\varepsilon = \varepsilon(t)$ were plotted for constant shear stresses of up to 12 dynes/$cm^{-2}$, velocity gradients of a practically steady course of $G = 10^{-6}$–$10^{-5}$ $sec^{-1}$ and temperature of +8°. The curve was plotted during 30–40 min.

The actomyosin gel-solutions have considerable thixotropy (Table 1). In view of the fact that the kinetics of thixotropic recovery of colloidal structure may depend on the velocity gradient of the initial destructive treatment [7], the preparation before relavation in the apparatus was standardly destroyed at $G = 150$ $sec^{-1}$ for 1 min.

The results of some of our experiments are shown in the Figures.

They show that during a week, the visco-elastic properties of the actomyosin gel-solution did not remain constant. Initially its moduli of shear $E_1$, $E_2$, $E$ (Fig. 1, A–C) and relaxation viscosity $\eta_1$ (Fig. 1, D) determined in the region $\eta_1 = \text{const.}$ of the curve $\eta_1 = \eta_1(P)$ [5], rose. On the 4th to 5th day, these parameters reached

their maximum, and after 5 days started to fall.* However, it should be noted that in some of our experiments, the fall off in the above-mentioned parameters for the actomyosin gel-solution had already started on the 2nd day after obtaining the preparation, this is possibly related to a variable degree of aggregation of the actomyosin particles in preparing it.

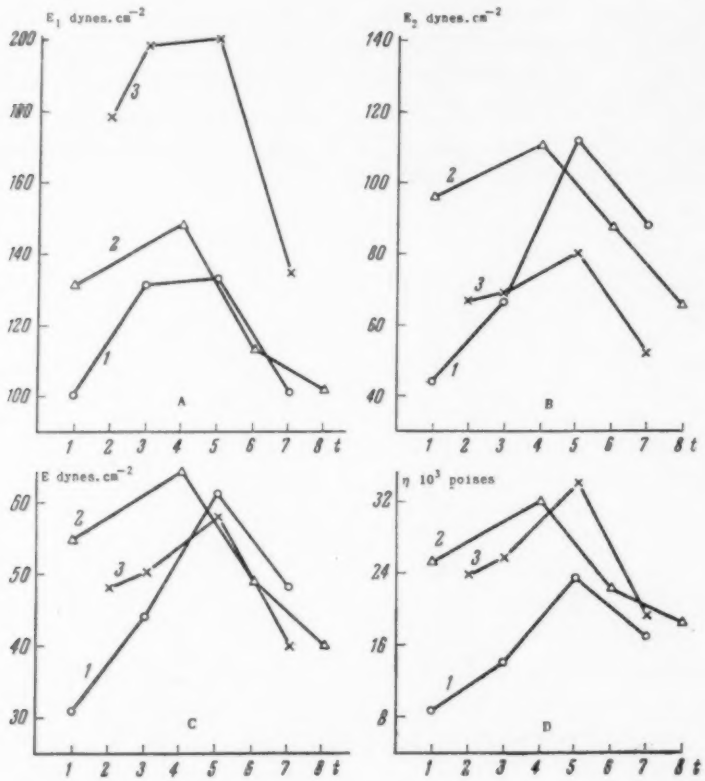


FIG. 1. Change in visco-elastic properties of gel-solutions of actomyosin on ageing for 1 week (explanation in text). Preparations from different rabbits; (1) protein concentration, $C = 2{\cdot}2\%$, time at rest in tube before measurement, $\tau_{rest} = 210$ min; (2) $C = 2{\cdot}4\%$; $\tau_{rest} = 250$ min; (3) $C = 2{\cdot}7\%$, $\tau_{rest} = 60$ min; $t$, time of ageing in days.

This suggests that during a week the actomysoin gel-solutions show continuous spontaneous structural changes which at first consolidate its structural network and then weaken it.

* This passage through a maximum of the structural-mechanical parameters of the colloidal system on ageing has recently been demonstrated on an object of non-biological origin — the gel of aluminium naphthenate — elements of the structural network of which constitute quasi-polymer particles formed in similar fashion to polyhydroxides [8].

Such structural changes may be associated with the changes in the interaction of the structural elements of the network between themselves and the dispersion medium and also with changes in the size and shape of the structural elements themselves.

The increase in the visco-elastic parameters of the actomyosin gel-solution is apparently due to an increase in the number of points of contact in its structural network, and conversely their fall to the decrease in the number of such points of contact. The latter is probably accompanied by a coarsening of the structural elements of the network and an increase in their length.

The author wishes to thank Doctor Trapeznikov for his interest in the work and valuable advice in carrying it out.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# STUDY OF THE STABILITY OF PROTEINS BY THE METHOD OF NONSTEADY HEAT EXCHANGE OF THEIR SOLUTIONS IN A HIGH FREQUENCY FIELD*

V. K. TKACH and L. A. FRENKEL'

Kharkov State University and the Institute of Medical Radiology, Kharkov

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IN a number of biophysical and biochemical investigations aimed at casting light on the stability of protein structures, the need for a study of the kinetics of dissociation of proteins, or conversely adsorption by them of the ions from the surrounding solution, has become apparent. The intensity of these processes gives an idea of the disturbances in the stability — chiefly expressed in a change in the surface (in this also sorption) properties of the proteins.

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Study of these processes also helps to disclose the patterns of change in stability of the blood proteins in various pathological states of the body [1, 2], and when exposed to various doses and forms of radiation [3–7]. A comparative study of protein dissociation and the adsorption of ions by proteins in solution by various methods, shows that a highly sensitive experimental method is the nonsteady heat exchange of protein solutions in a high frequency electrical field applied externally; this enables one to establish very precise criteria for evaluating the intensity of these processes. It is known that protein solutions are systems containing ions of variable dispersability and mass. Thus, if we consider a protein solution in water we can expect the presence in it of positive, negative and amphoteric ions of different size and charge. Ion formation may in a number of cases occur as follows. Even chemically pure water is partially dissociated into ions: $H_2O \rightleftarrows H^+ + H^-$. If we introduce a protein into the water the formula of which can be nominally written as: $R\begin{smallmatrix}NH_3OH\\COOH\end{smallmatrix}$, then because of the interaction of protein and $H^+$ and $OH^+$ ions the formation of new ions can be expected:

(1) $R\begin{smallmatrix}NH_3^+\\COOH\end{smallmatrix}$ (2) $R\begin{smallmatrix}NH_2\\COO-\end{smallmatrix}$ (3) $R\begin{smallmatrix}NH_3^+\\COO-\end{smallmatrix}$

In addition to this process of ion formation, it may be assumed that the protein molecules themselves (as amphoteric electrolytes) dissociate in water into acidic and basic ions: (the predominance of one type over the other dependent on the pH of the solution). The state of the ionic equilibrium in this case may be described as follows:

$$H^+ + \left[R\begin{smallmatrix}NH_3OH\\COO-\end{smallmatrix}\right] \rightleftarrows R\begin{smallmatrix}NH_3OH\\COOH\end{smallmatrix} \rightleftarrows \left[R\begin{smallmatrix}NH_3^+\\COOH\end{smallmatrix}\right] + OH^-$$

A characteristic feature of ion formation in this manner is the presence of four types of ions — ions of colloidal size bearing positive and negative charges and the $H^+$ and $OH^-$ ions of small mass.

Thus, while for globular proteins of an ellipsoidal shape the dimensions of the minor axes lie within the limits of 20–60 Å, and the lengths of the major axes within the limits of 40–200 Å, the $H^+$ and $H^-$ ions have radii 1–2 orders of magnitude less, for example, the radius of the non-hydrated $OH^-$ ion is 1·47 Å [8, 9]. If the hydration layers of the ions are taken into consideration, the difference in their dimensions and mass becomes even more significant. On introducing ions differing greatly in mass into an alternating electric field, it may be expected that their mobility and magnitude of displacement during a half-cycle of change in the field will show greater variation, the greater the difference in their mass. In fact, if in the first approximation we ignore the electrophoretic inhibition and relaxation phenomena which cause the dispersion of the ions, and consider only the inhibiting forces determined by Stokes law, then on applying to the solution an electric field with an amplitude of $E_0$ and a frequency $\omega$ acting on an ion with a charge $e$, the equation of motion of the ion may be written in the form:

$$m \cdot \frac{d^2x}{dt^2} + K\frac{dx}{dt} = eE_0 \cos \omega t,$$

where $x$ is the displacement of the ion, $t$ the time, $K$ a coefficient characterizing the interaction of ion and solvent (coefficient of friction) and $m$ the ionic mass.

Solution of the equation makes it possible to find the maximum displacement of the ion in the high frequency field and consequently the velocity of its motion:

$$V = \frac{eKE_0}{m(K^2+\omega^2)}$$

Knowing the velocity of motion of the ion, the size of its charge and the number of ions in one cubic centimetre ($N$), it is possible to find the density of the high frequency current of this particular ion in the solution:

$$I = NeV = \frac{Ne^2KE_0}{m(K^2+\omega^2)}$$

From the last equation the value of the electrical conductivity of the solution can be determined:

$$\sigma = \frac{I}{E_0} = \frac{Ne^2K}{m(K^2+\omega^2)}$$

If the solution contains ions of different mass then its total electrical conductivity is made up of the electrical conductivities of the ions of different mass and can be found from the following equation:

$$\sigma = \frac{N_1e_1^2K_1}{m_1(K_1^2+\omega^2)} + \frac{N_2e_2^2K_2}{m_2(K_2^2+\omega_2)} + \cdots + \frac{N_nn_n^2K_n}{m_n(K_n^2+\omega^2)}$$

In the present study we are concerned with a two-component solution containing large protein particles and mineral ions differing in the mass by several orders of magnitude. Therefore, in the electrical conductivity equation we may ignore all terms containing the masses of the large protein ions and assume that ions of the least mass, i.e. mineral, are chiefly involved in the electrical conductivity of the solution.

Therefore, the final value of high frequency electrical conductivity for the ions of the least, but roughly the same mass (with, of course, a certain degree of accuracy) can be written in the form:

$$\sigma = \frac{Ne^2K}{m(K^2+\omega^2)}$$

It may be assumed that the friction of the ions (value $K$) is proportional to their concentration in the solution. Therefore, the condition can always be chosen for which $\omega \gg K$, then

$$\sigma = \frac{Ne^2K}{m\omega^2}$$

That is, in the region of low ionic concentrations the high frequency electrical conductivity of the solution (for a given field frequency) will rise with an increase in the ionic concentration, and conversely, will fall for a decline.

The last equation indicates the possibility of creating experimental conditions in which even very slight changes in the concentration of the "small" ions may be measured by measuring the high frequency electrical conductivity of the solution or values proportional to it. Consequently, if the test protein undergoes dissociation in solution with formation of small ionized fractions or "simple" ions, this will cause an increase in the electrical conductivity. Conversely, the structural modifications in the protein causing increase in adsorption activity in relation to the "small" ions will involve a diminution in the electrical conductivity of the solution. To study the adsorption by proteins of the mineral ions of a particular type a definite ionic background is incorporated into the test solution, e.g. 0·01–0·1% NaCl.

The processes of protein dissociation and ion adsorption in a solution can be measured with the maximum facility and with relative simplicity of measuring apparatus if the high frequency electrical conductivity is measured during heating or cooling of the test solution. In this case we shall consider only the process of cooling.

As is known [10], the amount of heat emitted by a cooling body into the ambient medium during time $\tau$, and also the variations in its temperature, are determined by the equations:

$$Q_\tau = Q_0 e^{-k\tau}; \quad T_\tau = T_0 e^{-k\tau}$$

where $Q_0$ and $T_0$ are the quantity of heat and the temperature of the body initially, and $Q_\tau$ and $Q_\tau$ their values at time $\tau$ after the start of recording the cooling. The coefficient $k$ in these equations is equal to the ratio of the heat emitted by the cooling body to its heat content, i.e.

$$k = \frac{\alpha}{C} \frac{S}{M}$$

where $\alpha$, the coefficient of heat emission in cal $cm^{-2}$, $min^{-1}$, $degree^{-1}$, $S$ the area of the heat-emitting surface in $cm^2$, $C$ the specific heat of the body in $cal.g^{-1}$ $degree^{-1}$, $M$, the mass of the body in g. The coefficient $k$ has the dimension $\tau^{-1}$. It characterizes the rate of cooling (or correspondingly, heat liberation). The reciprocal of $k = \frac{\alpha S}{C M}$ has the dimension of time and is called the "time constant" of cooling of the body $\tau_b = \frac{1}{k}$.

Assuming that in a small temperature interval, the high frequency electrical conductivity of the solution varies linearly with temperature $\sigma_{t^\circ} = \sigma_0 (1 + \beta \Delta t^\circ)$, where $\Delta t^\circ = t^\circ - 18^\circ$ and $\sigma_0 = \sigma_{18^\circ}$ it is possible in determining the value $k$, and consequently $\tau b$, instead of measuring the temperature to measure the corresponding values of the electrical conductivity.

In fact, in the equation $\sigma_{t^\circ} = \sigma_0 (1+\beta\Delta t^\circ)$ we can substitute the temperature of the cooling medium: $\Delta t^\circ_\tau = \Delta t^\circ_0 e^{-k\tau}$, where $\Delta t^\circ_0$ is the selected temperature change during cooling and $\Delta t^\circ_\tau$ the temperature corresponding to different moments of time during cooling. Then $\sigma_{t^\circ} = \sigma_0 (1+\beta\Delta t^\circ_0 e^{-k\tau})$.

In the case of cooling, $\sigma_0$ is the value of the electrical conductivity of the medium at the start of cooling.

With an unchanged ionic concentration in the test solution throughout the time of cooling the values $\beta$ and $k$ remain constant.

The value of the rate of cooling can be derived from the last expression:

$$-k = \frac{1}{\tau} \ln \frac{\sigma t - \sigma_0}{\sigma_0 \beta \Delta t^\circ_0}$$

VOL. 5 1960

Thus, the nonsteady process of cooling occurring in a solution containing ions in a high frequency electrical field can be characterized by the parameters of the high frequency electrical conductivity; the latter correspond to definite moments of time and definite values of temperature. This in turn enables one to compute the basic characteristics of the process in question — the temperature coefficients of the high frequency electrical conductivity and the rate of cooling of the solution.

We shall explain in greater detail the relation of these values to the concentration of "small" ions in the solution.

If during a certain time interval the protein in the solution undergoes dissociation with formation of "small" ions, or, conversely, adsorbs them from the solution, then the concentration of these ions at the start and end of this time interval (and also the values of the electrical conductivity corresponding to them) will vary. Therefore, the values of the temperature coefficients of high frequency electrical conductivity at the start and end of the interval of time during which the concentration shifts occur, may be represented in the form of the expressions:

$$\beta_1 = \frac{1}{\Delta t^\circ} \cdot \frac{\sigma_1 - \sigma_{01}}{\sigma_{01}} = \frac{1}{\Delta t^\circ} \cdot \frac{\dfrac{N_1 e^2 k_1}{m\omega^2} - \dfrac{N_1 e^2 k_0}{m\omega^2}}{\dfrac{N_1 e^2 K_0}{m\omega^2}};$$

$$\beta_2 = \frac{1}{\Delta t^\circ} \cdot \frac{\sigma_2 - \sigma_{02}}{\sigma_{02}} = \frac{1}{\Delta t^\circ} \cdot \frac{\dfrac{N_2 e^2 k_2}{m\omega^2} - \dfrac{N_2 e^2 k_0}{m\omega^2}}{\dfrac{N_2 e^2 k_0}{m\omega^2}}.$$

The identical value for $N$ indicates that the completion of one cycle of cooling of the solution (for example, for a definite value $\beta_1$) takes such a short time that the dissociating or adsorbing protein "does not have time" to change significantly the concentration of the "small" ions in solution. Therefore, the changes in the electrical conductivity of the solution during this time are determined solely by the change in

the mobility and friction of the ions in the solution with variations of the temperature of the solution.

In carrying out a sufficiently rapid cycle of cooling of the solution, it is necessary to choose a temperature interval of cooling, the initial heat and the geometric parameters of the vessel containing the test solution.

In order to determine the difference in the temperature coefficients of high frequency electrical conductivity the equation may be written:

$$\Delta\beta = \beta_2 - \beta_1 = \frac{1}{\Delta t^\circ} \cdot \frac{\sigma_2 - \sigma_{02}}{\sigma_{02}} - \frac{1}{\Delta t^\circ} \frac{\sigma_1 - \sigma_{01}}{\sigma_{01}} = \frac{1}{\Delta t^\circ} \frac{\sigma_2 - \sigma_1}{\sigma_{01}} \text{ at } \sigma_{02} \approx \sigma_{01}$$

(the temperature interval, $\Delta t^\circ$, for each cycle of cooling remains constant). Substitution of the values $\sigma_2$ and $\sigma_1$ gives:

$$\Delta\beta = \frac{1}{\Delta t^\circ} \cdot \frac{\dfrac{N_2 e^2 k_2}{m\omega^2} - \dfrac{N_1 e^2 k_1}{m\omega^2}}{\dfrac{N_{01} e^2 k_0}{m\omega^2}}.$$

Assuming that the change in the number of ions in solution from value $N_1$ to value $N_2$ is such that it does not cause a serious change in the coefficient of friction of the ions, i.e. considering that $k_0 = k_1 = k_2$ we can obtain the final approximate expression:

$$\Delta\beta = \beta_2 - \beta_1 = \frac{1}{\Delta t^\circ} \cdot \frac{N_2 - N_1}{N_1},$$

since virtually $N_1 \approx N_{01}$. This equality also implies that the mass of the "small" ions is the same. The linear relation obtained between the relative changes in the concentrations of the "small" ions in solution and the changes in the temperature coefficients of high frequency electrical conductivity is well confirmed experimentally (provided that the difference $N_2$ and $N_1$ is not especially large).

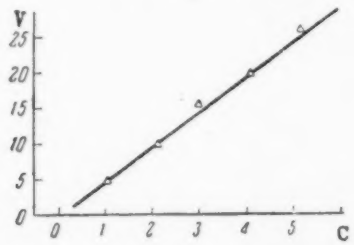


FIG. 1. Relation between the temperature coefficients of high-frequency electrical conductivity and concentration in solution. Ordinates, values of temperature coefficients of high-frequency electrical conductivity in arbitary units; abscissa, concentration of NaCl solution (value of each interval 0·0005 N solution).

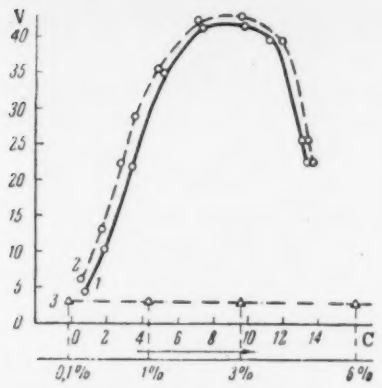


FIG. 2. Dependence of temperature coefficients of high-frequency electrical conductivity on concentration of protein and electrolyte in solution. Ordinates, values of temperature coefficients $V$ in arbitary units; abscissa, concentration of NaCl solution (each interval 0·001 N) and protein concentration in percentage; (1) NaCl solution, (2) NaCl + 3% solution of human serum albumin; (3) aqueous solution of human serum albumin without ionic background.

Fig. 1 shows the variation in the values of the temperature coefficient of high frequency electrical conductivity with concentrations of ions of NaCl in aqueous solution on change in the concentration from 0·0005 N to 0·0025 N. Over a wider interval of concentrations the values of the temperature coefficients change in the manner shown in Fig. 2 (curve 1).

The position of the peak of the curve of the values of this coefficient depends on the frequency of the external electrical field. Introduction of a definite amount of protein into the solution with a mineral ion background does not change the shape of the curve (Fig. 2, curve 2). The picture obtained fully accords with the hypotheses advanced above. The temperature coefficient of electrical conductivity shown as a function of concentration of protein in a solution not containing the necessary ionic background is depicted in Fig. 2 (curve 3). From the Figure it follows that change in the content of protein in solution (in the limits of concentration indicated) does not cause any fundamental change in the value of this coefficient.

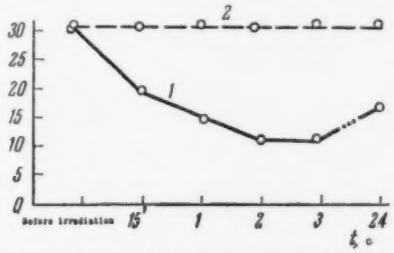


FIG. 3. Change in temperature coefficients of high-frequency electrical conductivity of protein solution with time after irradiation with $^{60}Co$. Ordinates, values of temperature coefficients $V$ in arbitary units; abscissa, time after irradiation in hr; (1) irradiated protein (3% human serum albumin); (2) native protein.

As stated above, a change in the concentration of the "small" ions in solution may produce a different rate of nonsteady heat exchange (in this case a different rate of cooling). The latter arises from the fact that changes in high frequency electrical conductivity of the solution, the temperature coefficients of high frequency electrical conductivity, temperature, time and rate of cooling are interrelated:

$$\frac{\sigma_1 - \sigma_0}{\sigma_0} = \beta \Delta t_0^\circ e^{-k\tau}.$$

By arranging that during measurement $\sigma_1 - \sigma_0 = \text{const.}$, $\Delta t^\circ = \text{const.}$ and $\tau = \text{const.}$ it is possible to obtain a function for the rate of cooling of the solution for various values of $\beta$ and, consequently, for different concentrations of ions in solution;

$$k = \frac{1}{\tau} \ln \frac{\beta \sigma_0 A t_0^\circ}{\sigma_1 - \sigma_0}.$$

Experimental checking of the method outlined showed it to be highly sensitive to those disturbances in stability of the surface structures of proteins which lead to concentration shifts in the ionic backround of the solution composed of "small" ions. Since the resolving power of the method is adjusted to define a concentration charge of the order of 0·000146 g/l. for a frequency of $7 \times 10^6$ c/s (which is not the limit) this allows one to measure with great accuracy the kinetics of the processes of protein dissociation (and also the desorption by them of ions) and the processes of adsorption.

As a typical example illustrating the interesting possibilities of the method, we give the curve of the kinetics of adsorption by proteins of $Na^+$ and $Cl^-$ ions on $\gamma$-irradiation (Fig. 3). As test object we used a 3% solution of human serum albumin prepared in double distilled water with the addition, for producing the ionic background, of 0·2 ml saline per 10 ml. Irradiation was carried out from a GUT-400 unit with a dose rate of 4200 r/min. Dose 82,000 r.

As the Figure shows, in this case we are dealing with a rise in the adsorptive capacity of the protein macromolecules. The investigations carried out with other doses showed that the intensity of the sorption processes in the protein structures varied with the dose of radiation. However, the duration of these processes is a matter of hours, which points to the appreciable after effects developing in the protein. This problem will be discussed in greater detail in a separate paper together with a fuller description of the measuring apparatus.

## SUMMARY

Consideration of the nonsteady process of heat exchange of solutions of electrolytes of electrolytes and proteins in a high frequency electrical field applied externally shows the following.

(1) The process of adsorption by protein of ions from the solution or, conversely, their desorption, can be defined by measuring the temperature coefficients of the high frequency electrical conductivity, the rate of cooling of the solution and "the time constant" of cooling.

(2) These values make it possible to define concentration changes in the ions, on conversion to weight units, of NaCl up to 0·000146 g/l.

(3) This method can be applied to study the kinetics of the sorption properties of proteins when subjected to radiations.

*Translated by* A. CROZY

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# THE SORPTION THEORY OF CELL PERMEABILITY AND THE PRE-EXISTENCE OF RESTING POTENTIALS*


G. A. KURELLA

Faculty of Fundamental Biology, Lomonosov State University, Moscow


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To date the sorption theory of cell permeability as formulated by Nasonov and Aleksandrov [1, 2, 4] and developed by Troshin [3], has excluded the possibility of the pre-existence of a resting potential (r.p.). In this sense it is essentially a specific

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expression of the damage theory of Herman [5]. The selective accumulation of potassium in the protoplasm has been regarded as the result of the formation in the cytoplasm of a protein-potassium complex, and it has been considered that nearly all the cellular potassium is in a non-ionized state. The denial of the possibility of the pre-existence of a r.p. in the last analysis, has cast doubt on the validity of all the factual material obtained by the advocates of all the modifications of the membrane theory.

The membrane theory [6–8] resting on an enormous number of experiments with a high level of methodological thoroughness, considers the pre-existence of an r.p. as a reliably established fact. The r.p. the value of which is most intimately bound up with the concentration gradient of potassium between the cell contents and the extracellular medium, may pre-exist if the potassium, selectively accumulated in the cytoplasm, is in the ionized form, i.e. if the activity of $K^+$ in the cell is high. The large amount of selectively accumulated potassium can only occur if in the system considered conditions are created leading to the establishment of an equilibrium state, outwardly similar to the Donnan equilibrium, i.e. if the cell contains anions which for some reason or other cannot penetrate its boundaries, whereas the cations are able to cross its boundaries in both directions.

One of the possible reasons for the zero mobility of certain anions, may be the existence of boundary cellular structures with the properties of a semipermeable membrane, across which the protein anions cannot diffuse. These concepts provided the starting point of the work of Bernstein who was the first to postulate the existence of a cell membrane and thereby lay the basis for the membrane theory [9]. In this case the cell contents, in particular, the protoplasm, must be considered as an aqueous solution. The selectivity of the cell to $K^+$ ions, as compared with $Na^+$ ions, can then only be a function of the cell membrane irrespective of the specific nature of the mechanism sustaining this selectivity in the resting cell, and its instantaneous disappearance on excitation. Of the cations, practically only potassium is involved in the resting state and in producing conditions similar in character to the Donnan equilibrium. This is despite the fact that the concentration of sodium ions in the extracellular medium greatly exceeds that of the potassium ions. Subsequently, with the help of labelled atoms, it was established that the potassium and sodium ions can freely traverse the boundaries of the cell in both directions. The latter, together with other factors, led to the notion that a membrane "sodium pump" exists. However, the introduction into current usage of the concept of membrane pumps has so far yielded no definite information on the nature of the processes responsible for selective cell permeability.

The membrane theory denies the possibility of the direct participation of intracellular structures in the processes ensuring the selective accumulation of potassium ions, merely assigning to the cell contents the rôle of a probable supplier of the energy required to sustain the activity of the membrane "pumps".

The results of experiments carried out by us with the intracellular microelectrode technique on isolated fibres of the sartorius muscles of grass frogs [10–12], have led us to conclude that the asymmetric distribution of ions between the cell and the medium

is determined, not by the properties of the cell membrane, but by selective sorption of potassium by the protoplasm. We were also able to satisfy ourselves of the pre-existence of a potential difference — recorded by means of intracellular microelectrodes from the resting fibres of the muscle.

It has been considered hitherto that the theories are mutually exclusive [13, 14]. What is the real position? In our view, the question is essentially whether the emergence in the cell of the ionic equilibrium described above, similar in character to Donnan equilibrium, is possible (with selective permeability of the cell for potassium) if there is no membrane present at the cell surface with selective permeability for the potassium ions.

The results of our experiments and an attentive study of the literature on cell permeability, the nature of the biopotentials, and certain branches of electrochemistry, have led to the working hypothesis that the selective sorption of certain ions by the protoplasm may occur according to the same general principle as the selective binding of opposed ions by certain polyelectrolytes, in particular, by such strong ones as ion-exchange resins.

VOL. 5 1960

It is commonly held that the opposed ions in the polyelectrolyte phase are in the main electrochemically active, i.e. behave in this respect in almost identical fashion to ions in aqueous solutions [15–19]. With such a mechanism of sorption the opposed ions are bound not by covalent (as the damage theory postulates), but principally by ionic, bonds in a polyelectrolytic gel structure with fixed charges. Consequently, for such a form of binding the pre-existence of a r.p. becomes possible even for the sorption mechanism of selective accumulation of potassium in the cell.

Various variants of such ideas have been propounded in recent years by a number of investigators in different countries [20–24]. To our knowledge, no direct potentiometric determinations of the activities of ions in such polyelectrolytic phases have been made.

To fix our ideas, it was first necessary to find a method of directly determining the activities of ions in the artificial polyelectrolyte phase. Preliminary model experiments of this kind were set up as a way of tackling this problem.

We used separate granules of the nonselective KU-2 cationic exchange resin, saturated with potassium ions and then washed off with water. Capillary microelectrodes of the usual type with a diameter of 3–10 $\mu$ filled with 3M KCl were inserted into these granules. Then the KU-2 granules, strung on the microelectrode, were passed through a number of 10-fold serial dilutions of potassium chloride solution.

The circuit thereby produced had the form:

| Saturated calomel electrode | Micro-electrode (KCl in water) | KU-2 ($K^+$ form) | KCl in water | Saturated calomel electrode | (1) |
|---|---|---|---|---|---|
| (—) | $[K^+]_1$ | $[K^+]_x$ | $[K^+]_2$ | [+] | |

The measurements were made with an electrometer valve [10, 11]. The following results were obtained:

TABLE 1

| KCl in solution | e.m.f. (mV) | Δ e.m.f. on 10-fold change in KCl concentration of ambient fluid (mV) |
|---|---|---|
| 3·0 M | — 1·2±3·4 | |
| 0·3 M | — 34·1±3·0 | 32·9 |
| 0·03 M | — 73·5±3·2 | 39·4 |
| 0·003 M | —107·1±3·6 | 33·6 |
| 0·0003 M | —143·5±5·7 | 36·4 |

Thus, the 10-fold change in concentration of the external solution led to a change in the e.m.f. on average by 35·6±2·9 mV. The control experiments, as expected, showed that replacement of the microelectrodes filled with 3M KCl by electrodes filled with 0·3M solution, led to a shift in the curve by about the same value, 35 mV. With an equal concentration of solution (in the microelectrode and externally) the potential difference was approximately zero (see Fig. 1).

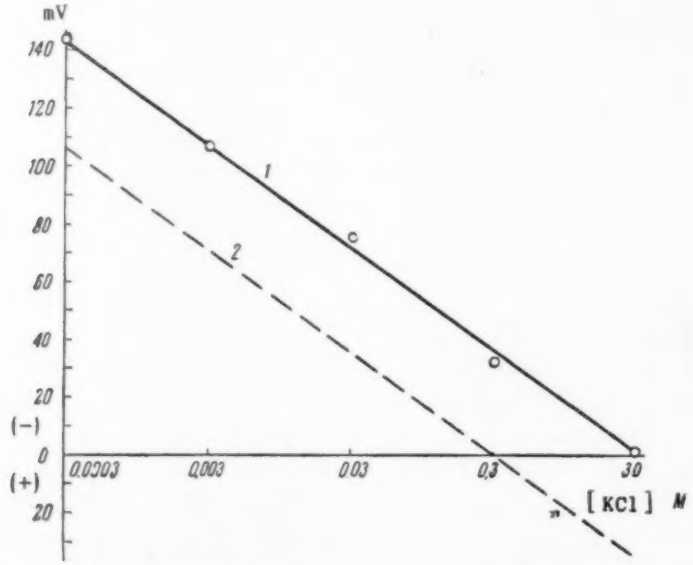


FIG. 1. Graph of variation in e.m.f. with potassium chloride concentration in external solution in circuit of type 1. (1) Microelectrodes filled with 3M KCl; (2) Microelectrodes filled with 0·3M KCl. Details in text.

These experiments convincingly showed that between the electrolyte in the microelectrode and the ion exchange resin phase, good contact was established.

Such types of circuits are practically identical with those used in electrochemistry for determining the activities of ions in solution for which reversible electrodes cannot be produced by means of ion exchange membranes [25]. They do not differ fundamentally from the liquid phase circuits. The rôle of the "non-aquous" phase in this case is played by the polyelectrolyte — the ion-exchange resin. The physico-chemical aspect of this problem has been discussed in detail in the specialized literature [26, 27].

The size of the e.m.f. developing in such circuits is determined by the Donnan potentials at the boundaries of the ion-exchange resin, and by the diffusion potential difference which depends on the mobility of the penetrating ion in the resin phase. The activity of the ions in the resin phase itself in these conditions remains unknown; in such cases it is immaterial to the activity of the ions determined in the test solution.

This circuit to a certain extent is similar to that proposed by Nasonov and his associates as an illustration of thier ideas regarding the mechanism of production of the r.p. after inserting a microelectrode into the muscle fibre — assuming the r.p. to be due to damage.

| Non-polarizing electrode | Protoplasm, altered or in state of paranecrosis | Intact protoplasm ("non-aqueous phase") | Lymph or Ringer solution | Non-polarizing electrode | (2) |
|---|---|---|---|---|---|
| (—) | $[K^+]_1$ | $[K^+]_x$ | $[K^+]_2$ | (+) | |

where $[K^+]_1 \gg [K^+]_2$

Comparison of the results of these model experiments with the published data obtained on muscle fibre [10, 12] suggests the following. Whatever theory we may hold — the membrane or the damage — the r.p. can be measured by means of intracellular electrodes only if the method excludes the possibility of the ions escaping indirectly to the electrode, since on damage to large areas of the muscle fibres [11], the r.p. vanishes within about 1 min. Apparently this is related to the equalization of concentration between the cell and the external solution. Obviously, in the muscle fibre as also in the granules of the ion exchange resin, the cone-like shape of the microelectrode provides reliable sealing of the entry aperture and prevents escape of theions.

In accordance with the concepts of the damage theory of the r.p. this might be, as mentioned above, merely the result of the disintegration of the protein-electrolyte complex on damage to or excitation of the cell. In either case the result should be the emergence of circuits of the phase above described, type (1). The similarity between the model experiments discussed and the circuits described by Nasonov *et al.* is also augmented by the fact that (as is visible under the microscope in the course of the experiment) insertion of the microelectrode into the granule of the ion exchange resin also destroys the structure over a small area; which in this case must lead to loss by this segment of its sorption properties.

In view of the above observations, the question naturally arises as to whether the value of the r.p. measured in the cell depends on the concentration of the solution

filling the microelectrode. Segal [28], sharing to a certain degree the views of Nasonov on the origin of the r.p., assumes that with microelectrode recording the cause of the appearance of the r.p. may reside in the fact that in the experiments microelectrodes filled with solutions of potassium chloride greatly exceeding in concentration the $K^+$ ions in the solution surrounding the cell are used. The value of the e.m.f. recorded from analogous models with an ion exchange resin, does in fact depend on the concentration of the solution in the electrode. However, checking showed that in work with cells, this concentration is practically irrelevant. The value of the r.p. recorded from the intact cell is virtually independent of the concentration of the solution in the microelectrode. It suffices to recall that recently we have used intracellular microelectrodes filled with Ringer solution of the same composition as that for the test preparation. The mean value of the r.p. recorded with these does not differ from the mean values obtained with electrodes filled with 3M KCl.

Comparison of the results of model experiments and those on muscle fibres is not in favour of the damage theory of the resting potential.

On the basis of the experience gained in the preceding series of experiments we produced circuits of the following type:

| Saturated calomel electrode | KCl in water (micro-electrode) | KU-2 K-from | Salicylaldehyde saturated with salicylic acid | KCl in water | KCl in water (micro-electrode) | Saturated calomel electrode | (3) |
|---|---|---|---|---|---|---|---|
| (—) | $[K^+]_1$ | $[K^+]_2$ | $[K^+]_x$ | $[K^+]_3$ | $[K^+]_1$ | (+) | |

Equilibrium of the system was reached after 12-48 hr. After this the potential difference recorded remained unchanged for a long time (in our experiments as long as 1·5 weeks).

On filling the microelectrodes with a solution of potassium chloride of the same concentration as the aqueous solution on the other side of the non-aqeous phase, i.e. when $[K^+]_1 = [K^+]_3$, the e.m.f. of the system was equal to $-27{\cdot}7 \pm 1{\cdot}5$ mV if the electrode was filled with 0·3M KCl (11 measurements during 7 days after establishment of equilibrium), and $-29{\cdot}9 \pm 1{\cdot}5$ mV when the microelectrode was filled with 0·003M KCl (seven measurements in 4 days).

In the corresponding phase circuits (1) and (4):

| Saturated calomel electrode | KCl in water | Salicylaldehyde saturated with salicylic acid | KCl in water | Saturated calomel electrode | (4) |
|---|---|---|---|---|---|
| | $[K^+]_1$ | $[K^+]_x$ | $[K^+]_2$ | | |

the e.m.f. was zero if $[K^+]_1 = [K^+]_2$.

In these experiments (circuit 3), the activity of the ions in the ion exchange resin phase was also dependent on the activity of the ions in the aqueous solution with which this phase was in equilibrium — in this case on the concentration of the solution

filling the microelectrode. However, this relationship was different from that in the previous experiments (circuit 1). Apparently, the reason for this is that the ionic activity measured by the above method (circuit 3) in the ion exchange resin is determined by two factors: (a) the capacity of the ion-exchange resin, depending on the number of charges fixed on the structure (for the cationites — negative charges), to bind electrostatically the equivalent number of opposed ions (sorption); and (b) on the activity of the ions in the solution occupying the so-called dynamic space in the ion exchange phase, i.e. that part of the total volume of the swollen resin in which the water is not bound and can serve as a solvent. At the moment of equilibrium in this space the activity of the ions will correspond to their activity in the equilibrium solution. The proportion of the total volume of the polyelectrolyte phase assigned to the dynamic space will depend on the degree of swelling of the polyelectrolyte gel. The degree of swelling in turn may depend quite appreciably on the concentration of the equilibrium solution [15–17].

VOL. 5 1960

For the KU-2 cation exchange resin it is known that the number of negative charges fixed on the structure, related to the dry weight of the resin, is almost constant and varies little with the pH and the concentration of the equilibrium solution. The equivalent number of opposed ions sorbed by this phase and maintaining the electroneutrality of the polyelectrolyte phase is also practically constant. If after establishing equilibrium with any solution we replace it by a solution of the same salt, but of different concentration (both being less than the concentration of the fixed anions) then initially there will be change in the activity of the ions in the dynamic space with a resulting change in the general overall activity of the ions in the entire polyelectrolyte phase. As for the change in the degree of swelling depending on the conditions of the newly established equilibrium, it will depend on the characteristic of the polyelectrolyte, and is not very important for KU-2. For others, especially polyelectrolyte gels which swell markedly, these changes may, however, be quite important, and even in the case of the relative independence of the degree of dissociation of the polyelectrolyte from these factors must lead to a sharp change in the proportion of the dynamic space to the total volume and, consequently, to a change in the total activity.

If the condition given above, i.e. when $[K^+]_1 = [K^+]_3$ (circuit 3) is satisfied, the activity of the ions in the dynamic space can be taken as equal to $[K^+]_1$ or $[K^+]_3$. Therefore, the potential difference recorded in these conditions apparently characterizes the activity of the sorbed fraction, i.e. the ions expended on neutralization of the fixed charges.

The results of all the series of experiments (circuit 3) can be summarized in the following Table (Table 2).

The e.m.f. values in the liquid phase circuits of type (4) are given in Table 3.

The results unambiguously indicate that the activity of the ions in the polyelectrolyte phase is much higher than in the equilibrium solution despite the fact that the opposed ions are "bound" in this phase by the ions of opposite charge fixed on the structure.

The first series of experiments discussed in the present article forces us to doubt the probability of the r.p. appearing as suggested by the authors of the sorption (damage) theory of cell permeability.

TABLE 2

| Experiment No. | $KCl_1$ (moles) | $KCl_3$ (moles) | e.m.f. (mV) | Number of measurements |
|---|---|---|---|---|
| 4 | 0·3 | 0·0003 | —87·2±1·25 | 11 |
| 3 | 0·3 | 0·0003 | —84·6±3·63 | 15 |
| 2 | 0·3 | 0·003 | —58·3±0·58 | 3 |
| 1 | 0·3 | 0·03 | —42·5±0·00 | 2 |
| 4 | 0·3 | 0·3 | —27·7±1·55 | 11 |
| 5 | 0·003 | 0·0003 | —62·2±2·19 | 7 |
| 6 | 0·003 | 0·0003 | —48·8±0·27 | 5 |
| 5 | 0·003 | 0·003 | —29·9±1·48 | 7 |
| 6 | 0·003 | 0·3 | +9·5±0·35 | 5 |

The second series of experiments suggests that the r.p. pre-exist given an accumulation of the potassium ions in the protoplasm (even in the presence of an excess of sodium ions in the external solution) — this is the result of their selective sorption and not a function of surface membrane mechanisms. However, these experiments, as follows from the theory of phase circuits, indicate that it would be impossible to measure the r.p. by microelectrodes inserted into the cell, if the cell were not bounded outwardly by a membrane or by some other dividing phase analagous to salicylaldehyde saturated with salicylic acid in our model experiments). Otherwise, the circuit formed would have the form of the circuits of type (1) or (2) and the measured e.m.f. (r.p.) would depend on the concentration gradient between the intraelectrode and the extracellular solutions.

TABLE 3

| Experiment No. | $KCl_1$ (moles) | $KCl_2$ (moles) | e.m.f. (mV) | Number of measurements |
|---|---|---|---|---|
| 4 | 0·3 | 0·0003 | —59·7±1·47 | 11 |
| 6 | 0·3 | 0·0003 | —57·9±0·24 | 5 |
| 5 | 0·003 | 0·0003 | —32·2±0·57 | 7 |

We assume that this may be a matter of boundary formation of the precipitation layer type described by Heilbrunn [29] (in the broad sense of the concept) and not having marked selectivity in relation to potassium ions against a background of a relatively high concentration of sodium ions in the equilibrium solution.

In order to prevent possible misunderstanding, a further factor must be dealt with. In our model experiments (circuit 3) the activity of the polyelectrolyte phase depended on the concentration of the solution in the microelectrode. However, with

the equality $[K^+]_1 = [K^+]_3$ the e.m.f. did not equal zero, as should have been the case, if the activity of the ions in the polyelectrolyte phase was not greater than that of the ions in the equilibrium solution or in dealing with circiuts of type (4) or (1). It has been pointed out above that equilibrium was reached in these experiments only after 12–48 hr. The reason for this is that diffusion from the microelectrode into the resin phase is limited by the narrowness of the capillary lumen. However, even compared with this, the diffusion of potassium ions across the salicylaldehyde saturated with salicylic acid diminishes slightly after equilibrium between the solution in the microelectrode and the resin phase, the activity of the ions in the latter continues to remain virtually constant. It is dependent on the concentration of the solution in the electrode which in this case also will be in equilibrium. In experiments on cells when much finer microelectrodes were used, with a diameter of less than 1 μ, the diffusion from the tip of the microelectrode was, in contrast, small as compared with the rate of diffusion of the potassium ions through the cell surface even if the microelectrodes were filled with 3M KCl [30]. It will be less, the smaller the concentration gradient between the extracellular medium and the contents of the microelectrode. Therefore, the cell contents are in equilibrium with the extracellular solution and not with the solution in the microelectrode, and the value of the e.m.f. measured is practically independent of the concentration of the latter. That is why even micro-injection of potassium ions into the cell does not lead to a change in the r.p. despite the expectations of the authors [31, 32], setting out from the premises of the membrane theory. At the same time, the removal of water from the cell by a hypertonic external solution [33] leads to an almost complete change in the value of r.p. since the shrinkage of the cell changes in the first place the fraction of the dynamic space in the total volume of the cell, and so leads to a change in the intracellular activity of the potassium ions.

Starting from the notion of the polyelectrolytic nature of the protoplasm, one can probably explain the fact that the cell does not behave like a osmometer [4]. The distribution of ions between the cell and the external solution is due to the same principle as systems where the mobility of anions is equal to zero because of the existence of semipermeable membranes. Ionic equilibrium of the Donnan type is established in all cases where one ion has zero, i.e. in all cases in which, due to the zero mobility of one of the ions, the ions of the opposite charge are distributed asymmetrically because of the need to maintain electro-neutrality in each phase. The potential difference between these two phases is the result of the unequal distribution of ions. If an equilibrium of the Donnan type is established at the membrane, then the closed cell must behave almost like an osmometer. If this equilibrium occurs in the system, polyelectrolyte gel — equilibrium solution — then instead of osmotic pressure we shall be dealing with the so-called swelling pressure which differs fundamentally from the osmotic, although certain analogies may be drawn between them [15].

Support for the polyelectrolyte nature of the protoplasm is also afforded by the experiments of Simon *et al.* [21, 24], who established a parallelism between the entry of sodium and chloride ions into the cell, and also by a number of studies on the course of exchange of labelled potassium, sodium and chloride between the cell and

the external solution [24, 34]. These lead to the conclusion that in the cell there must exist "two volumes", one, freely exchanging ions with the external solution is apparently no other than the dynamic space, the existence of which is characteristic of ion exchange resins and most polyelectrolytes.

Apparently, the resting cell must be considered as the heterogenous phase of strongly-swelling, combined polyelectrolyte gels with an overall specific selectivity for certain ions, in particular, potassium ions. This phase forms at its surface the precipitation layer (in the formation of which a fundamental rôle, among others, must be assigned to the calcium ions [29]). The selectivity of the system $K^+/Na^+$ in the resting fibres of the sartorius muscle, judging from the results of quantitative determinations, must be of the order of $1 \times 10^2$–$1 \times 10^3$. The fraction occupied by the dynamic space in the total cell volume is a variable quantity and depends on the level of the exchange sustaining the metastable structure of the combined polyelectrolyte gels of the protoplasm (protein, polysaccharides, nucleoproteins, etc.). In this dynamic space ("free dissolving volume"), the ionic concentration is proportional to that in the external solution (*in vivo*, lymph). According to Simon *et al.*, for the surviving preparation of the sartorius muscle of the toad, it can reach one-third of the total volume of the muscle. This is the probable explanation for the large disparity in the data on the intracellular concentration of sodium ions — related usually to the entire volume of the cell. Indeed, sodium is, in the main, represented in the dynamic space, the size of which depends on a multiplicity of factors chiefly of a metabolic order [21, 24].

The main and fundamental difference between artificial polyelectrolytes and protoplasmic-structured polyelectrolyte gels is their relative stabilities. The protoplasm of the living cell at rest in the steady state has a definite specific structural organization, to the extent that the specific metabolism is not disturbed. The slightest action can lead to a marked change in the whole labile structure — its transformation (excitation, contraction, etc.) or to death of the cell.

It seems to us that the concepts briefly and very broadly outlined here are in good agreement with the experimental findings of the supporters of both prevailing theories on the origin of the r.p. — membrane and sorption. Possibly this brings us closer to a solution of the problem of the mechanism of cell selectivity in other words, to the nature of "sodium and other pumps", transposing the site of action of these from the cell surface to deep within the protoplasm where, as is known, all the leading metabolic processes occur. Perhaps these ideas will also help to bring nearer the solution of the problem of the relation between biopotentials of muscles and their contraction.

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VOL
5
196

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OL. 5 960

# EFFECT OF TEMPERATURE ON THE FAST AND SLOW POTENTIALS OF THE RHYTHMICALLY ACTIVE MUSCLE FIBRE, WITH INTRACELLULAR RECORDING*

A. I. SHAPOVALOV

First Medical Institute, Leningrad

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THE rhythmic activity of the muscle fibre produced by calcium deficiency, consists of slow potentials spreading with a decrement of negativity waves and fast spike potentials [1]. To elucidate the mechanism of the origin of the fast and slow oscillations of cell polarization, it is important to investigate their sensitivity to a number of external stimuli.

## METHODS

The experiments were performed on isolated preparations of the sartorius muscle of the grass frog. The muscle was placed in a chamber with modified Ringer solution* from which Ca ions were either absent of reduced to 10 per cent of the normal value. The muscle was stretched on a cork plate so that its length was increased by 25–30 per cent of he resting state value. In such conditions the probability of appearance of rhythmic activity is maximum [1]. The temperature of the preparation was changed by replacing the solution surrounding the muscle with Ringer solution heated or cooled to the requisite temperature; this was measured by a mercury thermometer.

The muscle potentials were recorded by glass capillary intracellular microelectrodes filled with 3 M KCl solution. The resistance of the electrodes was 3–10 MΩ, the diameter of the tip less than 1 μ. The microelectrode was attached to the grid of a cathode follower [2] connected to a d.c. amplifier. The output stage of the amplifier was connected to the vertical deflector plates of a cathode-ray oscilloscope. The potentials picked up were recorded by photographing the trace for a single sweep. In order to record the long rhythmic-acting processes, the final stage of the amplifier was attached, through a low ohmic output, to a MPO-2 loop oscillograph. Both the fast and slow potentials recorded by loop 5 (resistance 4·5 Ω) fully corresponded in shape, amplitude and duration to the oscillations recorded by the oscilloscope. Therefore, in most experiments the oscilloscope served for visual observation, and the photorecording was carried out by the tape winder of the loop oscillograph.

## RESULTS

In accord with the published data [1], the rhythmic activity of the muscle fibres at room temperature (18–20°) is distinguished by high variability. Some of the rhythmi-

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cally-active fibres generated spike potentials with prepotentials preceding them, whereas in many fibres slow waves of depolarization were observed (Fig. 1 (a) (b) and (c)). The slow waves often reached a considerable amplitude and duration. Since the slow waves represent local changes in he potential of the surface of the fibre, their amplitude cannot be accurately defined, as it depended on the distance between the site of puncture of the cell by he microelectrode and the point of origin of the slow wave. Nevertheless, it must be stated that the amplitude of the slow wave often

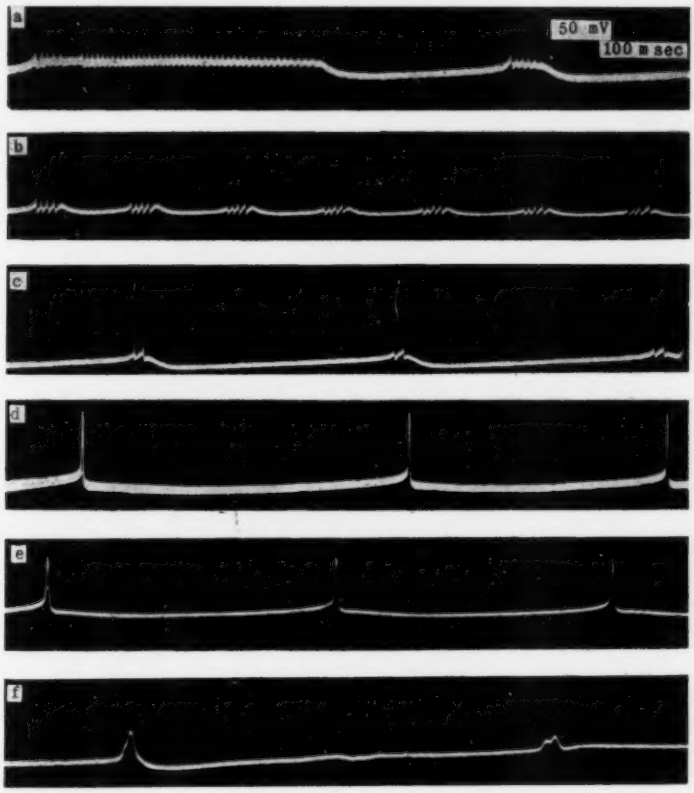


FIG. 1. Effect of cooling on the rhythmic activity of a muscle fibre (a) 20°; (b) 17°; (c) 14°; (d) 11°; (e) 7°; (f) 2°.

reached 30–50 mV. The duration of the individual slow oscillations greatly varied from fibre to fibre and frequently appreciably changed during the rhythmic activity of a given fibre. In some cases it reached 600–700 msec.

Very often at the apex of the slow wave there appeared spike potentials, the number of which was inconstant (Fig. 1 (a)). On the slow wave of long duration it was possible to count as many as 50–60 spike potentials, generated as a rule with a high frequency (on average 50–100 sec). However, in most cases the duration of the slow waves and

the corresponding number of spike potentials on it was much less. Nearly always the spike potentials formed at the apex of the slow wave of depolarization were preceded by separate small slow oscillations in potential—prepotentials. The prepotentials formed at the apex of the slow waves were similar to those preceding the single spike potential and differed from them only in the greater steepness of rise. Therefore, it may be assumed that the slow waves and the prepotentials are not identical and are oscillations in the polarization which may develop independently of one another.

In some fibres the slow waves, even of large amplitude and duration, were not accompanied by the appearance of spike potentials. In the course of the rhythmic activity the amplitude and duration of the succeeding slow wave tended to fall off, though in some cases the reverse was observed.

A fall in temperature had a non-uniform effect on the character of the fast and slow depolarization waves (see Fig. 1). Cooling by 3–4° made practically no difference to the frequency, amplitude and duration of the spike potentials of the given muscle, whereas the size and the duration of the slow wave appreciably diminished. In addition, the rhythm of the slow oscillations was reduced in frequency. At a temperature of 14–15° the slow waves were observed extremely rarely and when they appeared no more than two spike potentials ever developed at their apex. On cooling to 13–12°, the slow waves vanished completely (Fig. 1 (d)). At this temperature only the spike potentials and the prepotentials were recorded. The duration of the spike potential increased slightly. As the temperature was further lowered there was a progressive increase in the duration of the spike potentials, this is graphically represented in Fig. 2.

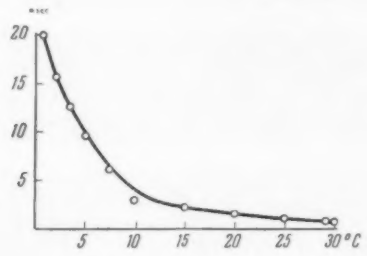


FIG. 2. Relation between duration of spike potentials and temperature. Ordinates, duration of spike potential in msec; abscissa, temperature.

There was little change in the amplitude of the spike potential on cooling to 11–10°, it then started to fall off rapidly. At a temperature of 3–4° it hardly ever exceeded the resting potential which in turn was reduced by 5–6 mV as compared with the normal. The frequency of the rhythmic activity tended to fall with cooling. The fall in the frequency of the spike potential started to become noticeable at about that stage of cooling at which the slow wave vanished. After lowering the temperature below 9–8° in most fibres displaying rhythmic activity the pulsed frequency was greatly reduced and was usually not more than 1–2 per sec. In addition, a noteworthy feature

of the rhythmic activity of the cooled muscle was the very rapid progressive decline in the frequency of the fibres showing rhythmic activity. Whereas at a temperature of 14–20°, rhythmic activity persisted for a long time, only gradually showing a fall in the frequency of the spike potential, on intensive cooling (3–8°), the distance between the succeeding spike potentials of the rhythmically-active fibre very quickly rose and the rhythmic activity ceased. The result was that at a temperature of 2–3°, the rhythmic activity was manifest in the appearance of only a few, usually not more than 1–3, spike potentials. At the same time, on moderate cooling (to 16–14°) the ability of the fibre to display rhythmic activity for a long time was increased.

A characteristic feature of the rhythmically active fibres cooled below 10° is the appearance of bifurcated spike potentials (Fig. 1 (f) and 3 (c)). The division of the spike potential together with a sharp increase in their duration undoubtedly indicates that the whole fibre is not excited at the same time. Apparently, the effect of cooling is to produce variable excitability in the various regions of the same cell with the result that the depolarization wave spreads along them at different speeds. A contributory factor may also be the retardation in the passage of excitation along the fibre always accompanying lowering of temperature. The split spike potentials substantially reduced in amplitude and with a long duration, as observed with severe cooling, differed little in their outward appearance from the local variations in potential.

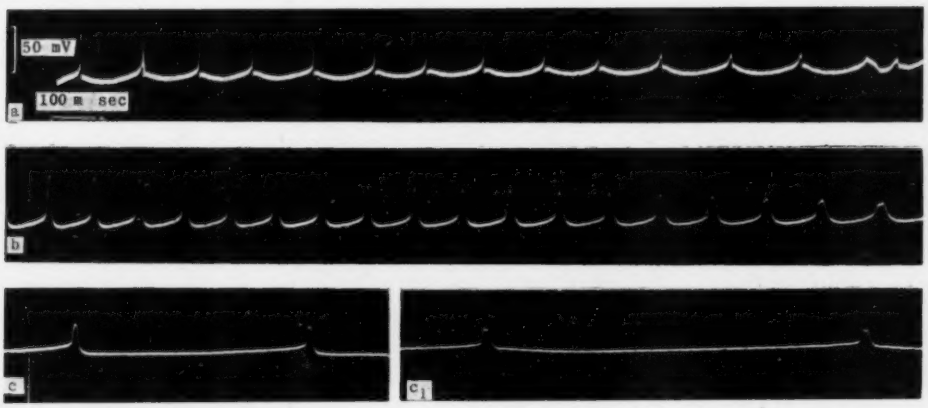


FIG. 3. Examples of rhythmic activity of muscle fibres with variations in temperature. (a) 23°; (b) 9°; (c) and ($c_1$) rhythmic potentials of same fibre at 6°.

It should be noted that whereas the process of lessening and disappearance of the slow wave and increase in the duration of the spike potential was strictly regular and found for all the rhythmically-active fibres, a reduction in the frequency of the rhythmic activity as cooling proceeded was sometimes absent (see Fig. 3 (a) and (b)). Unfortunately, the effect of cooling on the rhythmic activity of the individual fibre could not be elucidated since it ceased too rapidly. As for the different fibres of the same muscle, they could give a rhythm of activity uniform in frequency and amplitude

even for a temperature difference of 15–16°, although in the cooled fibre the duration of the spike potential was increased 4–6 times.

It thus follows that increase in the duration of the spike potential as such, and the attendant increase in refractoriness, are not the factors determining the decrease in the frequency of the rhythmic activity on cooling. The decrease in the frequency of the spike potential occurs as a result of the slowing down of the process of the rise in potential. Where the steepness of rise of the prepotential of the cooled fibre corresponds to that at normal temperature, the frequency hythmic activity is also similar. This interesting fact is in full agreement with the previous observations on the prime significance of the rate of rise of the prepotential [1, 3] for the rhythmic activity frequency.

Heating the muscle to the initial temperature, rapidly and completely restored the original properties of rhythmic activity. A rise in the initial temperature above room temperature (in the same way as the muscle was cooled) led to an increase in the amplitude and frequency of the slow waves and a shortening of their duration. Already, at 25–26° there was a sharp rise in the number of fibres in which rhythmic activity was manifest in the form of slow waves. Independent spike potentials, not superimposed on the slow waves and generated independently of them, were observed in these conditions only in a small number of fibres. The size of the spike potentials appearing on the slow waves was usually reduced as compared with those at normal temperature. The amplitude and duration of the spike potentials appearing outside the slow waves were also reduced (Fig. 4). A further rise in temperature resulted in the appearance in most fibres of slow waves not accompanied by a spike potential or the spike potentials which developed at the apex of the slow wave were very small — hardly distinguishable — representing "abortive discharges". At 29–30° the spike potentials vanished and the rhythmic activity consisted exclusively of slow waves. The resting potential of the muscle fibres then started to fall off rapidly, with progressive depolarization of the muscle fibres and heat contracture. Cooling (if the rise in temperature did not lead to irreversible changes) restored the changes induced by heating.

Thus, the results obtained point to a fundamental difference in the effect of temperature on the slow waves and on the spike potentials and their prepotentials. The slow waves vanish rapidly on cooling while the spike potentials continue to show little change. With intense cooling the spike potentials are maintained, although their duration and the steepness of rise of the potentials preceding them are greatly retarded. A different mechanism of formation of the slow waves, on the one hand, and of the spike potentials with their prepotentials, on the other, may thus be assumed. Accordingly, it is interesting to consider the relation at constant temperature between the slow waves, the prepotentials and the spike potentials.

Comparison of the rhythmic activity of different fibres at a temperature of 18–19°, shows that the size of the slow waves basically affects the amplitude and frequency of the spike potentials (Fig. 5). In the fibres with different resting potentials, the amplitude of the spike potentials varied, not with the level of the resting potential, but with

the amplitude of the slow wave at the apex of which the spike potentials. formed. Higher amplitudes of the slow wave were matched by a greater frequency and smaller amplitude of the spike potential, despite the fact that the level of the resting potential of the fibres for large slow waves may considerably exceed the level of the resting potential of the fibres with small slow waves. This fact is in accord with data obtained by artificial polarization of the rhythmically active muscle fibre with two concentric intracellular microelectrodes [3]. In addition, it underlines the fact that in determining the character of the activity of the muscle fibres it is not only the level of cell polarization in general that is of particular importance, but rather the value of the resting potential at which each spike originates. This conclusion is also confirmed by the

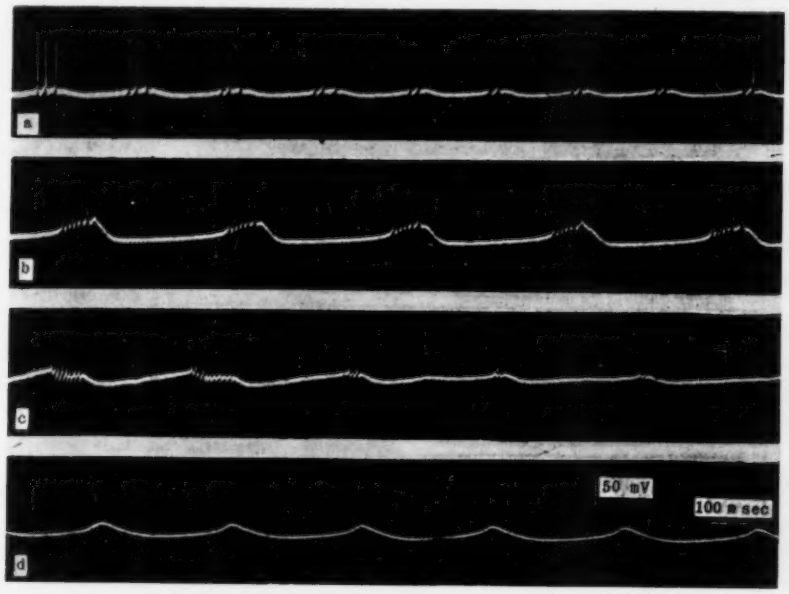


FIG. 4. Effect of rise in temperature on rhythmic activity of muscle fibre. (a) 18°; (b) 22°; (c) 26°; (d) 29·5°.

other records given in Fig. 5. The spike potentials arising at the start of the slow wave may exceed the amplitude of the succeeding spike potentials which appear against a background of a further rise in this slow wave (Fig. 5 (1) and (2)). In other cases, the amplitude of the slow wave rose so rapidly that already the first spike potentials formed on it were diminished as compared with those developing on the descending portion of the wave (Fig. 5 (4) (5) (6)). Finally, the spike potentials formed at an unchanged level of depolarization of the slow wave over a certain interval were of uniform amplitude (Fig. 5 (3)). The difference, in the amplitude of the spike potentials generated at different stages of the course of the slow wave, were so considerable that some (especially differing from others in amplitude) could be taken as discharges spreading from another part of the cell (Fig. 5 (7) (8)) since it was difficult often to

distinguish the prepotential preceding them [1]. In fact, perusal of this process at a high scanning speed, shows that the spike potential generated on the descending region of the depolarization wave may arise without any prepotential (Fig. 5 (6)), and this is true of both multiple and single spike potentials (Fig. 5 (9–12)). However, the absence of the prepotential in these conditions, in our view, does not necessarily

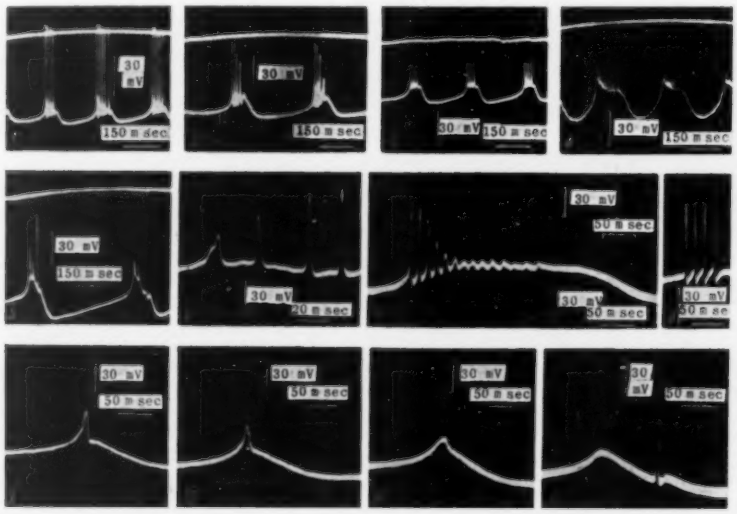


FIG. 5. Relation between slow and fast potentials of a rhythmically-active muscle fibre.

imply spread of the spike potentials from another part of the cell and that they do not arise from the point at which the slow waves are generated. It may be assumed that the depressing action exerted by a considerable depolarization on the generation of the spike potentials weakens as the slow wave diminishes and the already achieved level of depolarization provides the impetus which leads to the development of the spike.

Hence, the slow waves of depolarization may exist independently of the spike potentials but at the same time are very often the cause of the origin of the spikes and always exert a fundamental influence on the frequency and amplitude of the spike potentials and the prepotentials associated with them.

## DISCUSSION OF RESULTS

The observations described show that the spike potentials and the slow waves vary in their sensitivity to changes in temperature. In the literature, there are references to the effect that such a difference exists between spike discharges and the negative after-potentials of the frog muscle fibre on stimulation of a motor nerve [4]. It is of interest that the prepotentials constituting, like the slow waves, slow changes in polarization of the fibre, do not essentially change on cooling and heating. Only the steepness of their rise changes, i.e. like the spike potentials, in the main, the duration of the prepotentials changes. The slow waves only exist within definite temperature limits:

on cooling to 14–12° they completely vanish whereas the spike potentials and their prepotentials are detectable on cooling down to 1–2°. Heating, which leads to inactivation of the rhythmic activity, in contrast disturbs the generation of spike potentials somewhat later than it does the slow waves. It may be assumed from these findings that the process of generation of the slow waves and spike potentials differs in character. In the view of Lorente de No [5], the origin of the rhythmic activity is linked with a decrease in the slow fraction of the resting potential of the membrane (*L* fraction, to use his term).

According to the concenption developed by him, the resting potential of the membrane consists of several fractions of which two basic ones are of the greatest importance: the fast (*Q*) and slow (*L*) fractions. The spike potentials and certain fast electrotonic processes may be considered as changes in the fast *Q* fraction, whereas the after-potentials and slow electrotonic processes refer to oscillations in the slow *L* fraction of he resting potential. The slow fraction of the resting potential in this case is heat-labile, and the fast one heat-stable, since it depends on variations in temperature to a much lesser degree than the slow one.

It may be deduced from these arguments that the slow waves are due to variations in the slow fraction of the resting potential and that the spike potentials and their prepotentials are associated with fluctuations in the fast fraction of the resting potential, and hence are of a different nature.

The slow waves (as with the negative after-potentials of the muscle fibres [4]) probably display greater sensitivity to temperature factors because they represent the motion of ions depending on the energy resources. This explanation is supported by observations [4] showing that the inhibitors of oxidative phosphorylation readily suppress the negative after-potentials in muscle fibres. At the same time the spike potentials depend far less on metabolism since they are caused by the motion of ions along an electrochemical gradient. Since the prepotential, like the slow waves, constitutes an electrotonic change in the polarization of the surface of the fibre, it probably has a very similar mechanism of formation to the spike potential. The similarity between the spike potentials and the prepotentials, besides similar sensitivity to temperature, is also indicated by the observations already mentioned: the prepotentials usually arise at the apex of the slow waves as do the spike potentials. It has earlier been shown [1] that the formation of rhythmic prepotentials and the spike potentials is suppressed on decreasing the sodium ions in the solution surrounding the muscle. All this suggests that the ionic mechanism of generation of the spike potential and prepotential have much in common.

The slow waves of the rhythmically-active fibre have an affinity with the post-synaptic potentials which, according to Lorente de No, are manifestations chiefly of the slow fraction of the resting potential [6]. According to our observations [7], the post-synaptic potentials of skeletal muscle in conditions of Ca deficiency are able, like the slow waves, to give rise to multiple rhythmic responses.

All this suggests that the ionic mechanism of generation of the slow waves, the rhythmic prepotentials and their spike potentials varies. The slow waves may be

related to variations in the slow heat-labile fraction of the resting potential, the spike potentials and the prepotentials to changes in the fast heat-stable fraction of the resting potential.

Moreover, the argument of Lorente de No [5] that a necessary condition fo· the generation of rhythmic activity is a fall in the slow fraction of the resting potential was not confirmed in our experiments, if we consider the prepotentials as variations in the fast fraction of the resting potential. Rhythmic oscillations sometimes developed independently of the slow waves.

Inspection of the graph (Fig. 2) of the dependence of the duration of the spike potential on temperature, shows great similarity in the effect of temperature on the character of the spontaneously forming spike potentials in calcium deficiency, and those induced by stimulation of the motor nerve in conditions of normal ionic composition of the ambient medium [4]. This is an interesting finding in that (according to the data in the literature [8]) the spike potentials of the nerve fibres display in conditions of calcium ion deficiency much greater sensitivity to temperature changes than in normal conditions. The absence of such a difference in our experiments suggests the presence of special factors in the generation of the spontaneous spike potentials which reduce their sensitivity to the depressing action of cooling. Such a factor facilitating the appearance of a spike may be the presence of a prepotential.

VOL
5
196

## SUMMARY

(1) Intracellular microelectrodes were used to study the effect of change in temperature on the rhythmic activity of the skeletal muscle fibres in conditions of calcium ion deficiency in the ambient solution.

(2) It has been established that unlike the spike potentials and the electrotonic prepotentials preceding them, the slow waves of depolarization possess extremely great sensitivity to temperature fluctuations and that they have already vanished at the stage of cooling at which the spike potentials remain almost unchanged.

(3) A fall in temperature to 10–8° was accompanied in the main by an increase in the duration, and a decrease in the frequency, of the rhythmic spike discharges. Further cooling led to a reduction in their amplitude. On heating, the reverse pattern was observed.

(4) Reduction in the frequency of the rhythmic activity on cooling was due chiefly to a fall in the steepness of rise of the preceding prepotentials.

(5) Consideration of the dependence of the rhythmic activity on temperature and also the interrelations between its slow and fast components suggest that different ionic mechanisms are at the basis of the generation of the slow waves, on the one hand, and the spike potentials and prepotentials, on the other.

*Translated by* A. CROZY

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# ULTRA-VIOLET ABSORPTION BY THE *SEPIA* GIANT AXON IN STATES OF REST AND EXCITATION*

L. P. KAIUSHIN, R. G. LIUDKOVSKAIA and I. P. SHMELEV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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INVESTIGATION of the ultra-violet (u.v.) absorption in the living excitable nerve fibre is a means of exploring the functional chemistry and structure of nervous processes. The application of monochromatic ultra-violet radiation in these cases makes it possible, by the selective absorption, to differentiate the changes in such biologically important groups of substances as proteins and nucleic acids. Only a few attempts have been made to apply the method of ultra-violet absorption to the study of chemical changes during nervous excitation.

Among the first, was the work of Hyden [1, 2] in which a reduction in the protein and nucleic acid content of the motor cells of the spinal cord after intensive muscular work, and of the sensitive cells of the ganglion of the cochlea after auditory stimulation, was observed. Absorption in the region of 280 mμ fell 3–5 times, and the absorption maximum in the region of 260 mμ disappeared in most cases. These investigations were carried out on fixed but unstained material. A similar method has revealed an increase in the RNA content of the neurones of the retina on light stimulation [3]. An attempt has been made to investigate the change in u.v. absorption in the excitable living nerve of the frog [4] with photoelectrical recording. The author found a 3–5 per cent increase in absorption during excitation in the region of 265 mμ. However, these results required further confirmation. Recent investigations [5] have shown rapid reversible changes in the u.v. absorption of the protein extract of excited, as compared with unexcited, nerves. Despite the prospects offered by such investigations little use has been made of them for the purposes indicated above.

In the present work we made an attempt to apply the method of u.v. absorption in order to analyse the structural and chemical changes in the living excitable axon.

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The test object was the cuttle fish giant axon (*Sepia esculenta*). The work was done at the Institute of Oceanology of the Academy of Sciences of the Chinese People's Republic in the town of Tsing-dow.

Ultra-violet absorption in the region of 250–285 mμ of the axon in a state of rest or excitation was investigated, together with study of the effect of *u.*v. radiation of low intensity on the absorption by the nerve fibres.

The carefully dissected axon (diameter 200–300 μ) was placed in a chamber with a quartz base and secured to the stage of a MUF-3 u.v. microscope. The axon was stimulated with square pulses of current at a frequency of 50 c/s for $10^{-4}$ sec. The state of excitability of the nerve during photography in u.v. light was controlled by the action currents. The source of radiation was a SVD-120A lamp. A set of photographs was made of each axon with alternation of rest and excitation (magnification ×80). The photographs were taken in u.v. light with a wave length of 250–285 mμ, isolated by a combination of a gas (Cl, Br) and UFS-1 filter, and in the spectral region 270–280 mμ, using a combination of UFS-2 and gas filters. In the subsequent record the absorption curves were evaluated photometrically and compared. The exposures in the region 250–285 mμ lasted 5–10 sec, in the region 270–280 mμ, 2–2·5 min.

The photographic method has certain advantages (possibility of accumulation of the changes, allocation of the changes in absorption to definite structures, averaging of fluctuation of light) but gives comparatively large errors which must be borne in mind in evaluating the final results.

The error introduced by photometry and other factors can be estimated on repeat measurements of the same photograph or successive photographs with a uniform state of the nerve. This error does not exceed 10 per cent.

The direct action of u.v. radiation may influence the absorption of light by the axon. However, the effect only becomes manifest after prolonged irradiation of the order of tens of minutes; when adjacent frames are compared it is insignificant and consequently, does not increase the error of the method.

## RESULTS OF EXPERIMENTS

Sometimes it was possible to observe that a well-focused image of the axon at rest was out of focus during stimulation. The fine structural formations of the axon were orientated and elongated in a transverse direction as though the nerve had experienced small vibrations in this direction. Figure 1 shows photographs of the axon at rest (a) and during stimulation (b) taken in u.v. light at 250–285 mμ. In the latter case the image of the axon was out of focus and underwent transverse micro-displacement. This phenomenon was clearly visible in u.v. light. It may be compared with our observations on the frog sciatic nerve, where with the interference microscope in reflected visible light, elastic micro-oscillations during excitation of the nerve were demonstrated [6, 7].

In most of the experiments, stimulation of the axon did not result in visible structural oscillations of this type. Comparison of the photometric curves of the nerve at rest and in a state of excitation in these cases shows changes in the u.v. absorption. These

changes in the cases considered were from 15 to 25–30 per cent, i.e. considerably in excess of the experimental error. The changes in absorption were most often and clearly marked in the marginal regions of the axon embracing the membrane and the adjacent layer of axoplasm and constituting 15–25 per cent of he radius of the fibre.

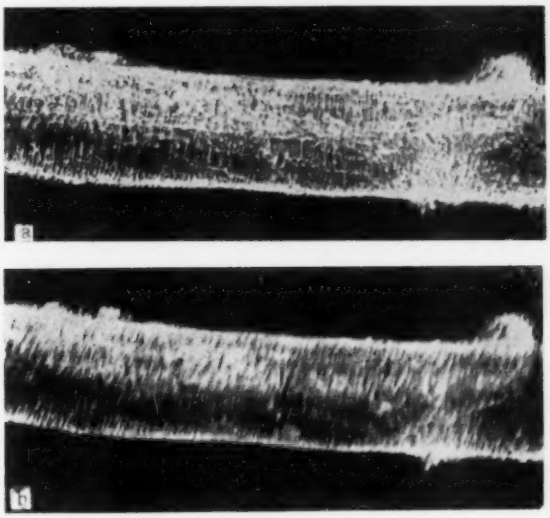

FIG. 1. Appearance of oscillations and defocusing of axon during stimulation. Photographed in u.v. region 250–285 mμ. (a) Giant axon a t rest. (b) During excitation.

In many cases the changes in absorption were visible not only in the marginal regions of the axon but over the entire diameter. In the central part of the axon they stood out more sharply against the structural heterogeneity.

The most clear-cut results were obtained with photography in a narrow region of the u.v. with maximum transmission in the region of 275–280 mμ. On stimulation of the axon, an increase in absorption was observed in these cases.

In the wider region of the spectrum, from 250–285 mμ, (maximum transmission 260 mμ) the absorption in the excited axon either rose or was less than at rest.

However, the shifts in the absorption curves in all these cases were quite distinct and were not less than 15–25 per cent of the resting value. It should be noted that the absorption effect was observed chiefly in the nerve fibres with stable excitability and in a good functional state.

At first this process was reversible and after ceasing stimulation the absorption curve returned to the resting level. But after several repeat photographs in u.v., the reversibility of the process disappeared. Sometimes it was possible to note that the absorption level at rest was not immediately restored, but that several seconds after switching off the stimulating current the absorption continued to shift in the same direction as during stimulation.

Monochromatic u.v. radiation incident on the axon during the photography cannot in itself affect the ability of the fibre to absorb light. This was more clearly manifest after repeated exposures. The direction of the absorption shifts varied for different axons. In most cases during an experiment lasting some tens of minutes, irrad ation produced a gradual lowering of the absorption. In other cases an increase was observed. Some axons were very resistant to the action of the u.v. and during the experiment their absorption did not change.

It was clear that in the majority of axons, the shifts in absorption due to stimulation and the effect of u.v. were of opposite direction, i.e. when the u.v. radiation reduced absorption excitation raised it, and vice versa.

This fact helps us to distinguish with greater reliability the absorption changes in the excitable nerve.

Long-acting u.v. radiation in addition to absorption changes caused a fall in excitability and a reduc ion in the action currents of the axon. However, the damaging effect of the various regions of u.v. on the axon varied. It is significant that u.v. radiation with a wavelength of 275–280 mμ (UFS-2 + gas filter) exerted a more injurious effect on the axon than did radia ion covering a wider portion of the spectrum — from 250 to 285 mμ (UFS-1 + gas filter) and of much greater intensity. This paradoxical fact had been noted by us previously in study of the effect of u.v. on the action current of the axon [8]. It may explain to a certain extent the high uniformity of the sorp ion shifts in the excitable nerve detected in the narrow region of the spectrum between 270–280 mμ as compared with the non-uniform effects in the region 250–285 mμ.

## DISCUSSION

The increase in the absorption powers of the excitable axon in the region of 275–280 mμ was associated with the accumulation of free chromophore groups selectively absorbing u.v. in this region. This phenomenon may be related to the dissociation of proteins accompanied by the splitting off of certain aromatic amino-acids, or by liberation of chromophore groups previously bound in the proteins of the axoplasm. In any case the process of formation of additional chromophore groups is the result of structural transformation of the protein molecule. The appearance of such effects in the excited axon indicates that the structural transformation of protein is of importance for the process of excitation, and in any event, accompanies a prolonged state of excitation in the axon.

The changes in absorption of the nerve in a wider u.v. range of the spectrum were less uniform though just as marked. In addition to the experiments in which the absorption of the nerve on stimulation increased, there were just as many in which a decline was observed. The absorption ability of the nerve in these cases was determined by the content of nucleic acids and proteins and their interaction. It may be assumed that over a wider region of the spectrum a change is recorded in at least two groups of substances.

Another possible process, in addition to that discussed above, is obviously associated with the transformation of the nucleic acids which have an absorption maximum

in the region 255–260 mμ. The enzymatic degradation of the nucleic acids leads to intensified absorption by reason of the hydrolysates involved. The strength of the link between the nucleic acids and the proteins and the action of the u.v. on this link, combined with effects connected with the degree of polymerization of the nucleic acids themselves, may exert an opposite influence on the absorption characteristics of the molecule.

The insufficient monochromatic nature of the u.v. radiation in our experiments, rules out a quantitative analysis of the results and may account for their lack of uniformity. However, these defects did not prevent us from establishing absorption changes in the nerve during excitation and the relation between them and definite groups of substances.

Attention must again be drawn to the comparatively high lability of the marginal zone of the axoplasm of the nerve fibre. However, this layer is quite wide and cannot be equated with the concept of a membrane characterized by electrical parameters. It seems to us that these findings serve as evidence of the differences in functional significance of the central and marginal zones of the axoplasm.

VOL. 5 1960

## SUMMARY

(1) Ultra-violet absorption of the *Sepia* giant axon has been investigated photographically in the region 250–285 mμ in a state of rest and excitation.

(2) The absorption powers of the axon in the region 275–280 mμ rose by 15–30 per cent on excitation. This is suggestive of accumulation of certain cyclical amino-acids or liberation of their chromophores, i.e. is associated with the structural reorganization of the protein molecule.

(3) The absorption of u.v. in a wider region of the spectrum (250–285 mμ) on excitation of the axon does not follow a uniform pattern: in some cases it increases, in others diminishes. The lack of uniformity in the changes may be the result of the interaction of two differently directed processes occurring in the nucleic acids and proteins.

(4) The change in the u.v. absorption of the axon during stimulation was observed principally in the marginal regions of the axon embracing a layer of thickness equal to 15–20 per cent of the radius. This indicates that the axial and marginal zones of the axoplasm differ in their rôle in the structural-chemical processes connected with the function of the axon.

(5) In some cases during stimulation elastic oscillations clearly demonstrable in u.v. light were observed.

We wish to express our deep gratitude to Prof. Feng De-pei and his colleagues for their interest and co-operation in the work.

*Translated by* A. Crozy

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* There is a translation into English of this work.

# ANALYSIS OF DISTRIBUTION OF POTENTIALS AND CURRENTS WITHIN THE RETINA ON LIGHT STIMULATION—II. PASSIVE DISTRIBUTION OF POTENTIALS AND CURRENTS WITHIN THE RETINA AS IN A VOLUME CONDUCTOR*

A. L. BYZOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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IN a previous paper [1] it was shown that the source of the currents forming the potential variations in the "nerve" layers of the frog retina lies in the bipolars of two types. Their activity is reflected in the two components of the e.r.g. recorded at different layers of the retina. A third component is recorded at the external limiting membrane (e.l.m.). What is the origin of this variation in potential?

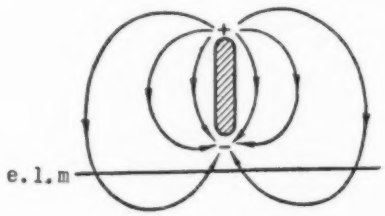


FIG. 1.

Since only the bodies of the rods and cones pass through the e.l.m., it is natural to assume, as we did earlier [2], that the source of the current forming this potential resides in the receptors. Subsequent observations have shown, however, that matters are more complicated. A notable fact is that the variation in potential at the e.l.m.

* Biofizika **5:** No. 3, 284–292, 1960.

broadly resembles the mirror image of the oscillations recorded in the nerve layers of the retina. When the latter change (for example as a result of changes in the intensity of the stimulating light) the negative oscillation at the e.l.m. correspondingly changes; and their latent periods are identical. All this makes us suspect that the variation in potential at the e.l.m. is formed as a result of the flow across it of the current generated by the structures of the nerve layers, i.e. by the bipolars of both types.

Such an "inflow" is easy to imagine (Fig. 1). If the dipole (bipolar) being the source of the extracellular current, is wholly situated to one side of the e.l.m., but sufficiently close to it, then through this membrane there must pass a certain proportion of current. In the areas away from the dipole the direction of the current across the e.l.m. is the same as in the nerve layers; in the proximity of the dipole, however, the current across the e.l.m. flows in the reverse direction. Since the resistance of the e.l.m. far exceeds that of all other layers [3, 4] this current may produce over a small region of the e.l.m. a considerable difference in potential which must precisely be the mirror image of the potential variations in the bipolar layer.

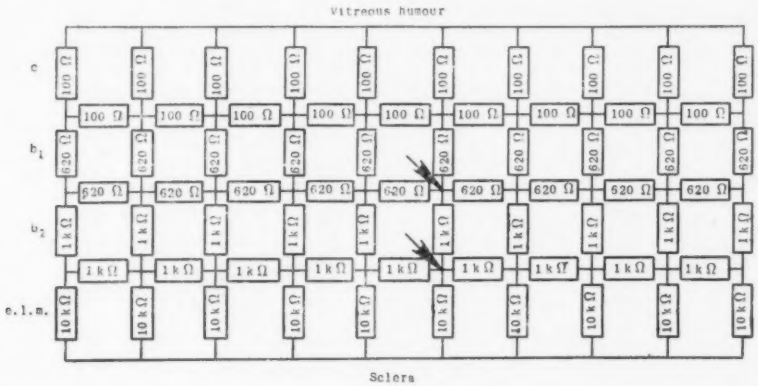


FIG. 2. Scheme modelling distribution of resistances in retina. Letters on left, corresponding to layers in retina; $c$, inner synaptic layer and layer of ganglion cells; $b_1$, inner zone of inner nuclear layer; $b_2$, outer zone of inner nuclear layer, outer synaptic and outer nuclear layer; e.l.m., external limiting membrane. Values of resistances given in kΩ. Arrows indicate points to which dipole connected if located on one of resistances of layer $b_2$ (see Fig. 3 (A) and (C)). Further explanation in text.

The distribution of currents and potentials in a volume conductor — retina — can be computed if we know the position of the dipole (dipoles) and the distribution in it of the resistances. However, we used a different, more convenient procedure — modelling.

In Fig. 2 is depicted a layout of the model reproducing in simplified form the passive electrical properties of the retina — the resistance of its different layers.* The model

* The capacity of the e.l.m. was not taken into consideration since its time constant is about 1 msec [4] and it cannot, therefore, play an important role in such relatively slow potential variations as in the case of the e.r.g.

comprises four "layers" corresponding to the inner synaptic layer (and the layer of ganglion cells $c$), the inner zone of the inner nuclear layer $b_1$, the outer zone of the inner nuclear layer, the outer synaptic and the outer nuclear layer $b_2$ and the e.l.m. The total radial resistance of each layer was proportionately increased 1·5–2 times as compared with the values measured on the retina experimentally. Thus, for layer $c$ it is

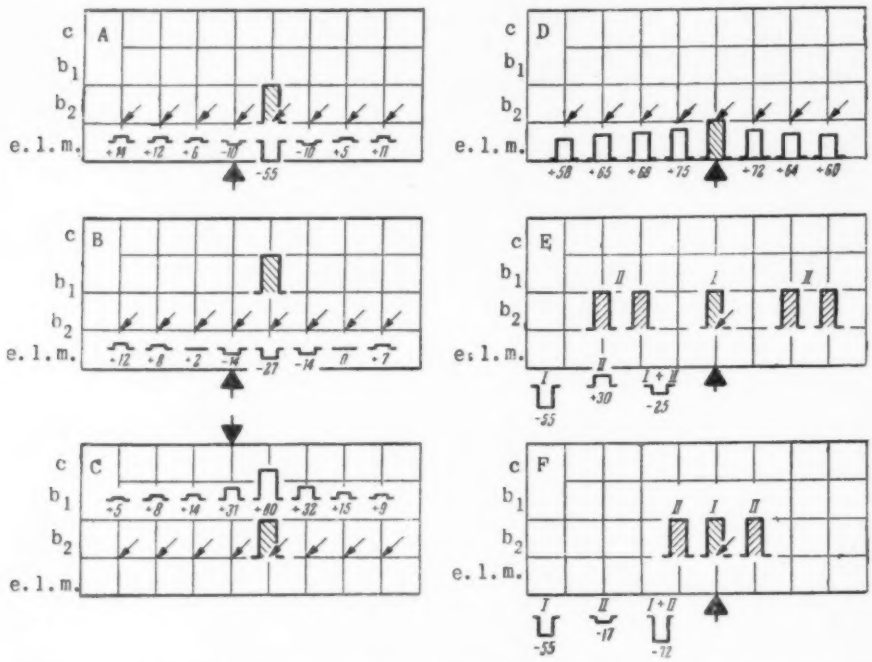


FIG. 3. Results of measurements of potentials on model for various positions of dipoles and recording electrodes. Graphs A–F reproduce different experiments on frog retina (see text). Sides of cells of each rectangle correspond to resistance of model in Fig. 2. Letters on left give layers of model as indicated in Fig. 2. Large arrows above or below rectangles indicate "macroelectrodes" located on "sclera" or in "vitreous humour" respectively. Small oblique arrows within rectangles indicate position of "microelectrode" for each measurement. Shaded columns indicate location of dipoles (voltage from battery over 5 kΩ). Unshaded columns together with numerals indicate potential difference (number of divisions on apparatus) measured for different positions of recording electrodes (A–D) or dipoles (E–F). On all graphs, the upward columns indicates positivity of upper electrodes. Columns below graphs E and F: I, potential difference at e.l.m. at switch on of dipole I; II same on switch on of two groups of dipoles II; I+II, same on simultaneous switch on of all dipoles I+II.

10 Ω, for $b_1$, 60 Ω, for $b_2$, 100 Ω, for the e.l.m. 1 kΩ [1]. The model is constructed on the premise that in each small volume of the retina the specific resistance in the radial and horizontal directions is about the same, and that in the horizontal plane the resistance is distributed more or less uniformly. From the point of view of the histology of the retina such an assumption is, on the whole, justified. All the upper and lower ends of resistances are interconnected. In the retina this corresponds to the vitreous

humour shunting, in fact, in short circuiting all the points of the inner surface and the layer of the outer and inner members of the receptors (and also the pigment and vascular layers and sclera) shunting the outer surface of the e.l.m.

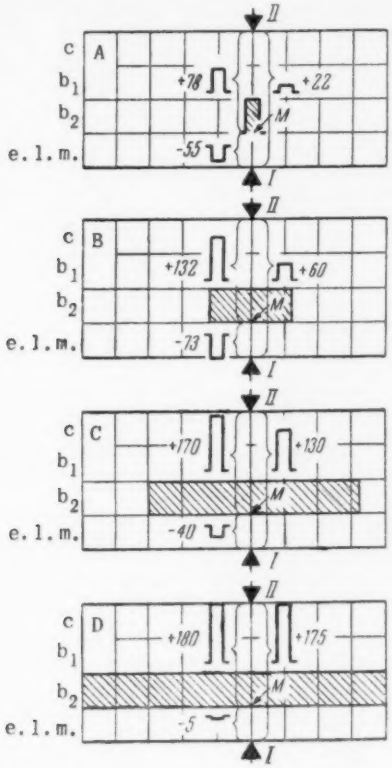


FIG. 4. Relation between "area of stimulation" and potential variations in different leads in the model. Shaded bands show over what resistances of layer $b_2$ dipoles in parallel were supplied. In each graph the downward columns on left indicate potential difference in lead I (between "microelectrode" and "sclera"); upward columns on left, in lead II (between "microelectrode" and "vitreous humour"); on right in lead I–II (between "sclera" and "vitreous humour"). M, microelectrode. Other symbols as in Fig. 3.

The "dipoles" (voltage supplied from batteries) were placed in layers $b_1$ and $b_2$ corresponding to the site of origin of the first and second components of the e.r.g. In order to avoid shunting of a given part of the model by the internal resistance of the batteries they were connected through series resistance of 5 kΩ. By supplying the voltage over one or simultaneously over several resistances (from individual batteries) it is possible to imitate the result of illumination of different areas and configuration, (e.g. an illuminated ring around the microelectrode). For measurement of the distribution of the potentials in the model, the recording electrodes connected to a sufficiently high resistance instrument were arranged in the usual manner for

experiments on the retina. One of the electrodes ("macroelectrode") was linked to the "sclera" (lower general lead) or to the "vitreous humour" (upper general lead) and the other to a particular point of the model, corresponding to the position of the microelectrode.

The scheme permits modelling of a large number of possible experimental variants. Here we shall describe the results only of changes which are comparable with experimental observations on the retina known from the literature or from our experiments.

Figures 3 and 4 give a simplified version of the model in the form of rectangles, the sides of the cells of which correspond to the resistances of the model in Fig. 2. The dipoles in layers $b_1$ and $b_2$ are denoted by shaded columns. The large arrows on the lower or upper sides of the rectangles represent the "macroelectrode" located on the "sclera" or in the "vitreous humour" respectively. The position of the "microelectrode" in each case is indicated by small oblique arrows within the rectangles. In all the experiments described in this paper this "microelectrode" was situated in the inner surface of the e.l.m. layer.

The potential difference measured at different positions of the recording electrodes is denoted in the form of unshaded columns together with potentials (number of divisions in apparatus). In Fig. 3 and 4 the upward direction of the column corresponds to the positivity of the upper electrode and the downward d rection to its negativity. Accordingly, we shal refer to the upward deviation as positive and downward as negative.

## COMPARISON OF RESULTS OF MEASUREMENTS ON MODEL WITH EXPERIMENTAL OBSERVATIONS ON RETINA

(1) In Fig. 3 (A) the dipole is positioned on one of the central resistances of layer $b_2$. In the retina this corresponds to excitation of the group of bipolars of the outer zone of the inner nuclear layer [1]. It is clear that on the e.l.m. under this dipole a potential difference of opposite sign to the dipole is recorded. It originates as a resul of "flow" of current from layer $b_2$ as shown in Fig. 1. A similar smaller negative variation at the e.l.m. was also recorded, when the dipole was located in layer $b_1$ (excitation of bipolars of inner zone of inner nuclear layer [1] as in Fig. 3 (B)). In principle, the same results were obtained also on supply of voltage over several adjacent resistances, which is analogous to stimulation by a light spot of a large area. Thus, the scheme gives a good model of the produc ion of the major part of the third component of the e.r.g. recorded at the e.l.m. (see above and al o [1]) and resembling the mirror image of the sum of the components recorded in the nerve layers.

(2) In the papers of Tomita *et al.* [5, 6] and Brindley [7–9], it is no'ed that on micro-illumination the po ential recorded by the microelectrode from the inner layers of the retina (the so-called "focal potential") is always negative irrespec ive of the position of the macroelectrode. It is clear from Fig. 3 (A), (B) and (C) hat the same thing occurs for the model. The circuit in Fig. 1 explains the reason for this: the microelectrode is located in the zone to which the lines of different current converge

and therefore it is always negative in relation to the sclera and the vitreous humour.

(3) The response of the retina to illumination by a microspot, recorded by the microelectrode from the inner layers is strictly local; it is only necessary to move the microspot by 0·5 mm, to sharply reduce it, or to eliminate it entirely [6, 7]. Such a local specificity of variation is also typical of the model (Fig. 3 (A) and (B)). This local dependence was somewhat less marked when the microelectrode was moved from the "sclera" to the "vitreous humour" (Fig. 3 (C)).

4) In those cases in which the greater part of the e.r.g. is formed at the e.l.m. (this is observed after a shift of the rod nuclei from the inner to the outer side of the e.l.m., for details see [2]) the potential variation was hardly localized at all. If the light spot is of sufficient size to produce a perceptible e.r.g. it is recorded not only in the illuminated area, but a considerable distance away from it.* Figure 3 (D) shows that this is the natural consequence of the passive electrical properties of the retina. Despite the fact that the dipole is located only on one of the resistances of the e.l.m. layer (source of current only in the illuminated region) a potential difference slightly less than the dipole itself is recorded well away from it on both sides.

VOL. 5 1960

(5) Brindley [7–9] describes an interesting and at first sight, not very intelligible observation. As stated above, the "focal" potential formed on micro-illumination of the region under the electrode is always negative. However, if we illuminate the retina with a ring so that the microelectrode is situated in its unilluminated centre,† then in certain cases a small positive variation is recorded. With simultaneous illumination of the centre and the ring the variation is intermediate between both cases. Figure 5 (A) depicts a similar experiment illustrating these points. Figure 3 (E) illustrates the same experiment for the model. On supplying the dipole to one of the resistances of the layer $b_2$ (microspot), a negative potential is recorded at the e.l.m. under dipole I (I, Fig. 3 (E) and Fig. 3 (A)). If two uniform groups of dipoles are located on both sides of the microelectrode at certain a distance from it (columns II, analogous to the illuminated ring) the potential variation is positive (II). Finally, simultaneous switching on of all three dipoles (illumination simultaneously of centre and ring) gives an oscillation intermediate between both cases (I+II at bottom of record). It must be stated that this result could easily be predicted from Fig. 3 (A) which shows that on moving the "microelectrode" to the side of the dipole (or on moving the dipole itself, which is the same thing) the potential difference at the e.l.m. changes its sign. Thus, the arrangement well describes the results of the Brindley experiment. However, more careful comparison of the results of measurements on the model with experiments conducted on the retina shows certain basic differences between them. On the model the oscillation obtained in the simultaneous presence of all three dipoles (I+II) was equal to the algebraic sum of the oscillations from

* This difference in local specificity of the "focal" potential and the total e.r.g. recorded at the e.l.m. was used by Brindley [7, 9] as an argument in favour of the conclusion that both these oscillations have their source in different structures of the retina.

† Second electrode on sclera.

dipoles I and II separately. In the retina, as Brindley has especially stressed, appreciable deviations from this rule are observed (Fig. 5 (A)). This is particularly clear in cases when illumination by the ring gives not a positive but small negative oscillation [7, 8]; on simultaneous stimulation by a microspot and ring the recorded potential is not the sum o the oscillations for the microspot and the ring separately, but an intermediate value between them. This result is inexplicable from the point of view of a purely

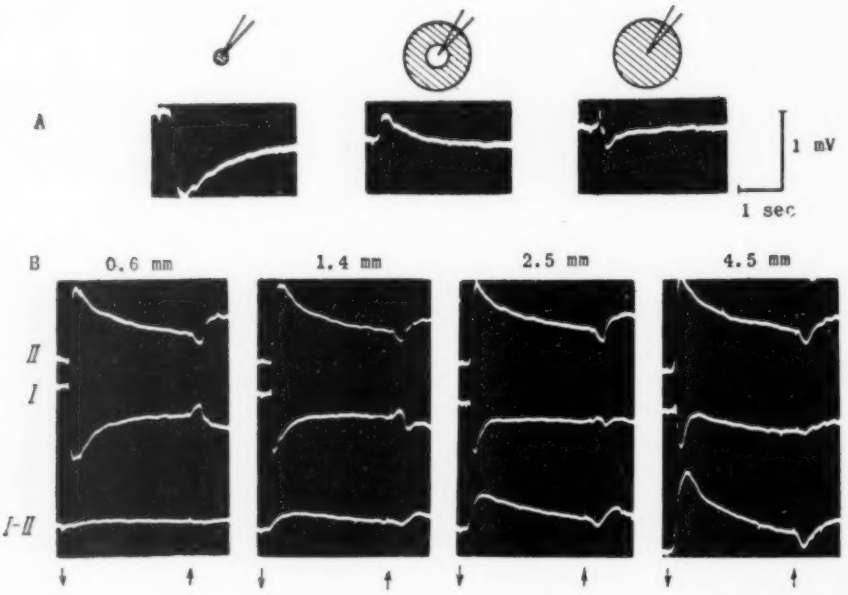


Fig. 5. Experiments on frog retina. A, potential variation in response to switching off light recorded by means of microelectrode for a different configuration of light spot (depicted above oscillograms). Microelectrode at depth of 180 μ from vitreous humour (close to inner surface of e.l.m.), second electrode on sclera. Diameter of microspot 0·6 mm, internal diameter of ring 1 mm, external diameter of ring (and also diameter of large spot on right) 4·5 mm. B, dependence of potential variations in different leads with area of stimulation. Numerals above columns, diameter of light spot. Microelectrode at depth of 200 μ, i.e. close to inner surface of e.l.m., second electrode on sclera. Lead II, between microelectrode and vitreous humour; lead I, between microelectrode and sclera; lead I–II, between sclera and vitreous humour. Arrows pointing downwards, moment of switch off of light, upward arrows, moment of switch on On all oscillograms in both A and B upward deviation indicates positivity of upper electrode. Experiments on isolated eye preparation at temperature of 10°.

passive distribution of currents since on the model the potential recorded on simultaneous supply of the dipoles I+II, in all cases was equal to the sum of the potentials on supply of dipoles I and II separately (Fig. 3 (F)). A plausible interpretation of this fact is given below.

(6) The proposed model also permits a certain degree of prediction of the results of certain new variants of the experiments. Figure 4 shows how the potential variations should change with an increase in the area of stimulation if these changes were depend-

ent solely on the passive distribution of currents in the retina acting as a volume conductor. The measurements were made in three leads: (a) between the "microelectrode" and "sclera" I; (b) between the "microelectrode" and the "vitreous humour" II; and (c) between the "sclera" and the "vitreous humour" I–II. The "microelectrode" was placed on the inner surface of the e.l.m. layer. The shaded bands show over what resistances the dipoles were supplied in parallel. For the sake of simplicity the dipoles were situated only in layer $b_2$.

As the number of dipoles (A–D) switched on in parallel increased, the potential variation in lead II (left upper columns) was more than doubled, in lead I (left lower columns) it increased somewhat and then fell heavily to almost zero in the case of D. In lead I–II, as to be expected, the potential difference increased appreciably and for each case was equal to the algebraic sum of the variations in leads I and II (Fig. 4).

Figure 5 (B) shows the same experiment on the frog retina. The microelectrode was lowered into the retina to a depth of about 200 $\mu$ so that its tip was close to the inner surface of the e.l.m.*

The figures above the vertical rows indicate the diameter of the light spot. The first horizontal row represents lead II (between the microelectrode and the vit eous humour), the second lead I (between the microelectrode and sclera) and third lead I–II, the total e.r.g. recorded between the sclera and the vitreous humour. Comparison of Fig. 5 (B) with the results of measurements on the model (Fig. 4) shows that for an increase in the area of stimulation the direction of the changes in both cases generally coincided. The only exception is lead I in which no increase, demonstrable by the model in the negative oscillation, was visible within the small areas of stimulation. However, it is clea· that wi h an unrestricted reduction in the light spot the negative oscillation in lead I as with the oscillations in any other leads, sooner or later must diminish and vanish altogether.

If we compare quantitatively the results of the measurements on the model and the retina, the first thing that attracts our attention is the following phenomenon. For the model there is a more than twofold increase in the oscillation in lead II as between case A and D whereas in the retina this increase is insignificant and in other experiments within the limits of the diameters of 0·6 mm to 2·5 mm the oscilla·ion even fell slightly. Thus, besides the passive distribution of currents and potentials there is another process at work here associated, apparently, with the interaction of the elements of the retina in the horizontal plane and leading to a reduction in the e.r.g. (for details see below).

## DISCUSSION

Hence, the model well reproduces the results of a number of experiments carried out on the retina. The number of examples of such agreement could be increased,

* The location of the tip of the microelectrode with respect to the e.l.m. was established by recording the potential variation for a pulse of current passed through the retina in a radial direction. For further details see [4].

for example, by varying the position of the light stimulation, the conditions of shunting (immersion of the whole preparation in Ringer solution), etc. Consequently, certain observations on the retina which are sometimes interpreted from the physiological point of view* may be ascribed to the purely passive electrical properties of the retina as a volume conductor. Tomita and Torihama [6] have also pointed to this factor.

On the other hand, in experiments on the retina, appreciable deviations are discovered from what should be observed given merely passive distribution of currents. Thus, in the above-described experiments of Brindley (see section (5)), the potential variation recorded on simultaneous illumination by a microspot and a ring, was considerably less than the sum of the potentials produced by the microspot and ring individually. A similar divergence of results from the expected (obtained on the model) was also observed in our experiments (see section (6)): the increase in the potential variation in lead II with an increase in the area of stimulation was, in fact, much less than predicted by the model.

Two suppositions may be advanced to explain these deviations [8]; (1) change in the resistance of the retina on illumination, and (2) the horizontal interaction between the nerve elements of the retina leading to their reciprocal inhibition. The first argument is inconsistent with the experimental findings since neither Brindley's experiments [3] nor ours revealed any appreciable changes in the resistance of the retina on illumination. The second assumption is very plausible if we remember the abundance of horizontal links in the retina, which are an histological fact, and also that the interaction between adjacent areas of the retina is clearly evident from the pulses in the ganglion cells [10, 11]. The problem of the interaction between adjacent elements of the retina requires more detailed study.

There are a number of other causes for the absence of a strictly quantitative agreement between the results of measurements on the model and experiments on the retina. They are primarily related to the fact that the values of the resistances in the different layers of the model were matched only approximately and a number of simplications had to be made. The true picture of the distributions of resistances in the retina is, in its details, much more complex. Further, in the retina on excitation the dipoles arise in both layers: $b_1$ and $b_2$ (excitation of bipolars of two types [1]); for the model, however, the dipoles are located either in one or the other layer. However, comparison of Fig. 3 (A) and (B) shows that in the two cases the distribution of potentials at the e.l.m. is, in general, similar. Therefore, on supplying the dipoles in layers $b_1$ and $b_2$ simultaneously the pattern of distribution of the potentials will, in principle, be the same as for a dipole situated only in one layer.

The reason for the differences between the measurements on the model and in the retina may also be he presence in the latter of dipoles not considered in the model. This applies firs ly to the horizontal amacrine cells which in conditions of micro-illumination, for example, can form horizontally orientated dipoles [7]. Secondly,

* For example, Brindley [7–9] relates the difference between the responses to local micro-illumination and to ring illumination (see above) to the activity of the horizontally orientated cells.

the source of the vertically orientated dipoles at the e.l.m. may be the receptors passing through this membrane. In the usual experimental conditions these dipoles, if they exist, are masked by currents with their source in the bipolars.

## SUMMARY

Measurements of the resistance of the individual layers of the frog retina served as the basis for designing an electrical circuit which models the properties of the retina as a volume conductor. In accordance with the results of our earlier work [1] the dipoles were produced over resistances corresponding to the "nerve" layers of the retina. It has been shown that the potential variation recorded by a microelectrode at the external limiting membrane and resembling the mirror image of the oscillations formed in the nerve layers of the retina [1] is connected in the main with the purely passive distribution of currents and potentials. This also explains a whole number of other peculiarities of the response to illumination recorded by several workers from the inner layers of the retina (for example, the local specificity of the response to micro-illumination [6, 7], inversion of the sign of the oscillation on replacing the microspot by an illuminated ring [7–9], etc). On the other hand, in experiments on the frog retina, certain deviations were found from what might have been expected from the passive distribution of currents and potentials (absence of additivity of oscillations recorded by means of the microelectrode [7–9]). It is assumed that this is related to horizontal interaction between the nerve elements of the ret na and leading to their reciprocal inhibition.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# PERCEPTION OF IMAGES OF GREAT BRIGHTNESS MOTIONLESS WITH RESPECT TO THE RETINA OF THE EYE*

A. L. IARBUS

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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IT is now well known, that within a test field motionless with re pect to the retina, all visible differences disappear and the "empty field" appears [1]. However, in all experiments of this type so far carried out the test fields have been of low or medium intensities. Therefore, it was interesting and important to find out whether differences in the test field vanish in these conditions if its component parts have blinding intensities.

The present paper is concerned with experiments with test fields of great intensity motionless with respect to the retina of the eye.

## METHODS

The test field was fixed relative to the retina by means of a suction disk similar to those previously described [1]. In the disk a small incandescent lamp connected to the supply source by very thin wires was assembled. The short-focus lens of the

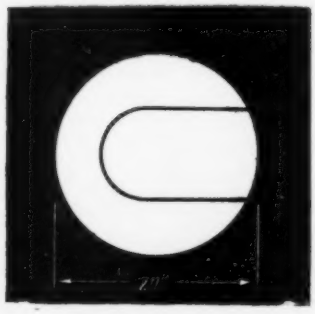


FIG. 1. Diagram of test field. Filament of incandescent lamp seen by subject through round diaphragm fixed closed to lamp.

disk gave a sharp image of the filament of the lamp on the retina. The pattern (test field) seen by the subject through the disk is schematically represented in the Figure. The thickness of the filament of the lamp was visible at an angle of 15′. By changing

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the voltage of the power supply, the brightness of the filament could be varied during the experiments. The greater part of the lamp was sealed with black paper so that no light fell on the sclera and therefore, when the experiments were carried out in a darkened room illumination of the eye via the sclera was completely excluded. After the whole construction had been placed on the anaethetized eye of the subject, the wires supplying the lamp were arranged so as not to interfere with the movements of the eyes nor cause the disk to shift on the eyeball. In some experiments in order to lessen the movements of the eye, the subjects were offered a fixation point which they looked at with the free eye. The whole construction (i.e. disk and lamp) did not exceed 0·4 g in weight. Four subjects took part in the experiments.

## RESULTS

(1) Our first concern was naturally to find out whether the filament vanishes for the subject against the background of the black field after the disk has been placed on the eye, i.e. after the image of the filament becomes strictly motionless with respect to the retina. The experiments showed that even for blinding intensities the filament vanished for the subject after 2–3 sec, i.e. the so-called empty field appears. If the second eye were covered by a black bandage a state occurred which the subject usually termed "complete darkness". If the other eye were open then the subject saw only what entered the field of vision of the second eye.

(2) It should be remarked that in conditions of high intensities the empty field appeared only after removal of a number of factors which change, even if only slightly, with time the distribution of light over the retina.

Usually to remove possible interference, atropine was injected into the conjunctival sac of the subject's eye before the experiment and during the test the experimenter carefully ensured that no extraneous factors caused deformation of the eyeball and displacement of the disk. One of the factors usually upsetting such experiments is illumination changing with time of the eye via the sclera. However, as stated already, in our experiments this interference was completely eliminated.

(3) In one experiment, after the disk had been placed on the eye the lamp was switched on at full power. During the first moment the subject saw a dazzlingly bright filament, then its visible brightness rapidly faded and the filament vanished completely after 2–3 sec (appearance of empty field). Later, it was found that the moment of switching off the lamp was also accompanied for the subject by the appearance of the dazzlingly bright filament which then completely faded out (complete darkness set in).

If, after the appearance of the empty field the brightness of the filament was varied step-wise each sufficiently sharp change (upwards or downwards) was accompanied for the subject by the appearance of the bright filament.

The differences within a test field unchanged and motionless with respect to the retina already cease to be perceived by the subject after 2–3 sec even when the separate parts of this field attain blinding intensities.

For useful discussion of the work the author wishes to express his deep gratitude to the Head of the Laboratories N. D. Niuberg and to the staff of the laboratory.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# COLOROMETRIC STUDY OF THE PROPERTIES OF COLOUR VISION OF INSECTS AS EXEMPLIFIED BY THE HOUSE FLY *

G. A. MAZOKHIN-PORSHNIAKOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

(*Received* 12 *March* 1959)

LITTLE is known about the spectral sensitivity of the eye of the house fly (*Musca domestica*) despite its availability as a specimen for experimental investigations and the notoriety of this insect in human epidemiology. Observing the behaviour of dark-adapted flies Cameron [1] noted that between 302–578 mμ, the fly is most strongly attracted by radiation with $\lambda = 365$ mμ. Donner and Kriszat [2] working with the electrophysiological method of R. Granit, determined the threshold curve of spectral sensitivity of the dark-adapted eye of this fly. The curve has two peaks: one at the violet end of the spectrum at $\lambda = 420$ mμ (not established very precisely) and the other, a lower maximum in the blue-green region from which the sensitivity smoothly falls to the red end of the spectrum. Finally, in order to establish whether the house fly has colour vision, Getsova and Lozina-Lozinskii [3] placed a coloured circle (made of paper) with a bait among three grey circles one of which "coincided in brightness with the circle of the test colou " (the authors do not say how they measured the brightness and whether it was for the eye of the fly or man). In experiments it has been found that flies exhibit a more distinct congenital positive response to green than to other colours, but they cannot be conditioned to a green reflex whereas this is quite easy in the case of red, yellow and blue. It is not hard to see however that with such an experimental design the result is not amenable to interpretation. We could only speak of colour vision in flies if they distinguished the circle of the test colour from any shade of another not necessarily grey.

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A more detailed study has been made of the colour vision of another synanthropic fly — *Calliphora erythrocephala*. The curve of the relative spectral sensitivity of the eye of *Calliphora* (this is the reciprocal of the number of quanta of the various monochromatic wave lengths giving the on-effect of the e.r.g. of equal height) has two peaks [4] and according to Walther and Dodt [5] three peaks one of which is in the ultraviolet. Walther and Dodt [5] are inclined to identify the number of peaks of the curve of spectral sensitivity with the number of independent light receptors of the eye. Autrum and Stumpf [4] recorded the e.r.g. and by substitution of monochromatic rays to give equal excitation of the eye (judging by the equality of their on-effect) found spectral colours distinguishable and indistinguishable for *Calliphora* between $\lambda = 400$–$700$ mμ. The colour vision of *Calliphora*, as these workers, assume, is dichromatic, however, they were not able to determine the curve of spectral sensitivity for each receptor.

VOL. 5 1960

In the electrophysiological experiments on the house fly described in this paper, we used the colorimetric method of studying colour vision, the application of which has made it possible to find the number and the shope of the curves of spectral sensitivity of the light receptors for the eye of man and certain animals [6, 7].

## METHODS OF INVESTIGATION

The object of colorimetric experiments is to measure the physical characteristics (the spectral composition and energy of radiations) which are indistinguishable (identical) by the eye; by varying these characteristics the properties of colour vision of the animal may be assessed. The colorimetric experiments were performed with a colorimeter — an optical instrument permitting visual comparison (to the point of complete indistinguishability) of any monochromatic radiation with a mixture of primary radiations ($A_1$ ..... $A_n$), i.e. to express spectral colour by a mixture of primary colours. By matching such visual equalities successively for several points of the spectrum and by measuring each time $\lambda$ and the relative energy ($E_\lambda$) of the monochromatic radiation being compared and also the relative energy of the primary radiations ($E_{(A_1)}$) .... $E_{(A_n)}$ it is possible to compute from the ratios $\frac{E_{(A_1)}}{E_\lambda}$, ..... $\frac{E_{(A_n)}}{E_\lambda}$ the functions $a_i(\lambda)$ and obtain the so-called "colour combination curves" for the given eye. The minimal number of linearly-independent primary radiations (correspondingly the number of functions ($a_i(\lambda)$), the mixture of which is visually indistinguishable from any monochromatic radiation directly characterizes the number of independent light receptors of the eye, i.e. the dimensions of colour vision. From the combination curves it is possible to estimate the wavelengths which the eye can and cannot discriminate, i.e. the diversity of colours existing for the eye. With a certain choice of spectral composition of primary radiations, the functions $a_i(\lambda)$ may directly turn out to be the curves of spectral sensitivity of the light receptors. In particular, if the primary radiations are so matched that each excites only one of the receptors, then the energy of the primary radiations $E_{(A_1)}$ .... $E_{(A_n)}$ will be proportional to the excitations of the receptors when the eye is acted upon by an indistinguishable (from the

mixture of primary radiations) monochromatic radiation $E(\lambda)$. In this case, the functions $a_i(\lambda) = \frac{E_{(A_1)}}{E_{(\lambda)}} \ldots\ldots a_n(\lambda) = \frac{E_{(A_n)}}{E_{(\lambda)}}$ will independently of one another characterize the sensitivity of each receptor to radiation $E_{(\lambda)}$.

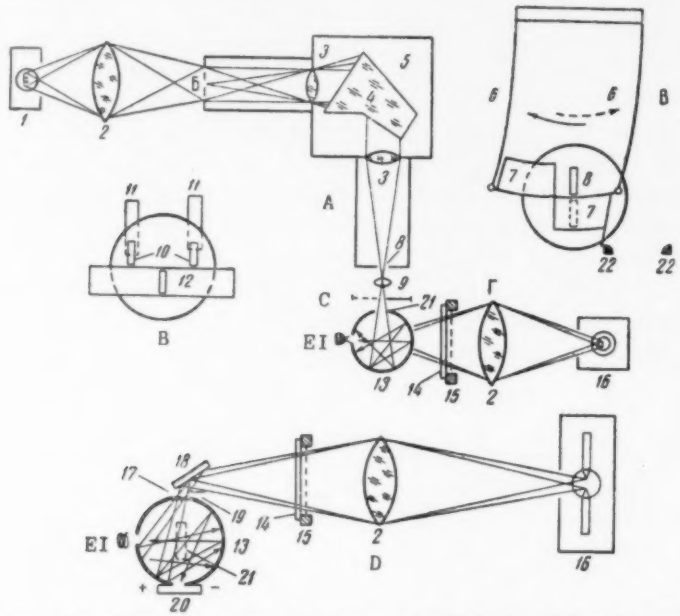


FIG. 1. Diagram of substitution colorimeter. (A) general arrangement of colorimeter (top view); (B) entrance slits; (C) main shutter; (D) mixing chamber and optical system with mercury lamp (side view); (EI) ege of insect; (1) incandescent lamp 400 W, 30 V; (2) condenser; (3) objective; (4) prism; (5) frame of monochromator; (6) springs of shutter; (7) slides of shutter; (8) exit slit; (9) lens; (10) upper entrance (primary) slits; (11) slide of slits regulating amount of light; (12) adjustable entry slit regulating $\lambda$ of measured radiation; (13) mixing chamber; (14) light filters for separation of $\lambda \sim 365$ m$\mu$; (15) diaphragm regulating u.v.; (16) PRK-4 mercury lamp; (17) upper aperture of mixing chamber; (18) mirror; (19) extra shutter, movement of which synchronized with movements of main shutter; (20) calibrated photocell; (21) side aperture of mixing chamber through which measured and primary radiations penetrated; (22) stops.

For the transition to an equi-energetic spectrum the relative energy of each radiation of the colorimeter is computed from the relation $E = \frac{\alpha_{(\lambda)}}{S_{(\lambda)}}$, where $\alpha(\lambda)$ is the galvanometer reading on illumination of the photocell by radiation $\lambda$, and $S_{(\lambda)}$ the sensitivity of the photocell to this radiation. Since $\lambda$ for each primary radiation is fixed, then $F_{(A)} = \alpha_{(A)}$.

The design of the colorimeter (Fig. 1) on which the experiments were carried out on the fly* has been described in detail in connexion with a similar investigation of

* In these experiments the source of the u.v. rays was the PRK-4 mercury lamp (instead of the SVDSH-250) and rays with $\lambda = 365$ m$\mu$ could be used as the primary or measured radiation.

the colour vision of the dragonfly [7]. Unlike the visual colorimeters usually used for the investigation of human vision, in our colorimeter, the colour fields to be compared were divided in time (substitution colorimeter [8]), i.e. by means of an adjustable shutter the radiation to be measured can be rapidly, in approximately 1/300 sec, and without a pause be replaced by the primary radia ion or by the mixture of several primary radiations.

The work of the eye of the fly was recorded electrophysiologically — from the e.r.g. formed on substitution of the radiations (Fig. 2). Where the radiation being measured and the mixture of primary radiations were not identical for the eye, the e.r.g. was visible on the screen of the oscillograph. By varying the intensities of the primary radiations a ratio could be achieved, for which on replacing the radiation to be measured by the primary (and vice-versa) the eye did give an electrical response

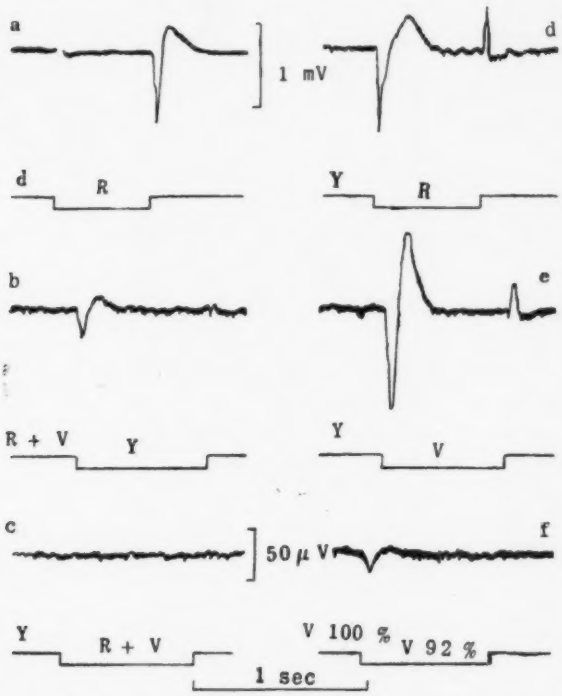


FIG. 2. Electroretinogram of house fly. (a) e.r.g. at switch on and off of red light; (b) e.r.g. in response to replacement of mixture of red and violet primary radiations by monochromatic yellow radiation (and vice versa) when both types of raidiation are visually similar for fly, but still not identical (insufficiently matched colour equality); (c) absence of e.r.g. with complete visual equality of yellow and mixture of red and violet; (d) e.r.g. in response to replacing yellow by red (and vice versa) which in mixture with violet was indistinguishable from yellow; (e) e.r.g. in response to replacement of yellow by violet (and vice versa) which mixed with the preceding red was indistinguishable from yellow; (f) e.r.g. in response to replacement of violet of intensity 1.0 by same violet of intensity 0·92 (and vice versa). (D) darkness, (R) red (630–680 mμ); (Y) yellow (600 mμ); (V) violet (420–455 mμ). Recording of e.r.g. in Fig. (a) was made with amplication 3 times less than for other figures.

(Fig. 2 (c)). The absence of an e.r.g. was taken as the criterion of the visual indistinguishability of these radiations for the eye of the fly. The accuracy of adjustment of the colorimeter and the reliability of the entire apparatus were estimated by the absence of an e.r.g. on substituting physically identical radiations, i.e. when the measured and primary radiations were physically equal.

The technique of leading off, amplifying and recording the e.r.g. has been described by us earlier [7]. Before the experiments the flies were rendered motionless by injecting ~ 0·005 ml of a 10% urethane solution.

The colorimetric experiments were carried out on 29 specimens (males and females) from flies bred in the Department of Entomology of the Moscow State University.

## RESULTS

The reaction of the fly's eye to a burst of monochromatic light over a very wide range of the spectrum is an electric response. For relatively high intensities of light, the sensitivity of the eye was investigated in the longwave region up to $\lambda = 710$ mμ and somewhat beyond, and in the short wave to $\lambda = 365$ mμ and even shorter, but owing to technical difficulties we were not able to investigate the region $\lambda < 365$ mμ. For relatively low brightnesses (twilight illumination) the spectrum visible to the fly is somewhat shortened — at the red end it reaches only 650 mμ.

For low intensities of light any monochromatic radiation visible to the fly can be matched to the primary radiation by suitably changing only the energy of the primary. In one series of experiments, such one-dimensional equalities were obtained for the primary radiation $\lambda = 420$–$455$ mμ, in another for the primary radiation $\lambda = 365$ mμ. The combination curves obtained as a result of measurement of the values $a_{(\lambda)}$ for each two primary radiations showed quite good agreement. In these experiments the contrast sensitivity of the eye of the fly was 3–20 per cent, i.e. there was a distinct electro-response on substitution of rays of uniform spectral composition differing in energy by 3–20 per cent (Fig. 2 (f)). The higher the contrast sensitivity of the eye preparation, the more well defined were the visual equalities. Therefore, the physical characteristics of radiations not distinguishable for a preparation with such relatively high contrast sensitivity quite precisely characterize the radiations which the fly cannot visually distinguish.

If we carry out an experiment with relatively high intensities, then one-dimensional equalities cannot be obtained over the entire spectrum. The eye of the fly does not distinguish any monochromatic radiation with λ shorter than 520 mμ from the corresponding intensity of the violet primary radiation ($\lambda = 420$–$455$ mμ); but for λ greater than 520 mμ it is no longer possible to select energies of measured and primary radiation, substitution of which, would not produce an e.r.g. However, a mixture of two primary radiations, violet and red ($\lambda = 630$–$680$ mμ) with certain matching of the energy of each cannot be discriminated by the fly from all monochromatic radiations with λ from 520 to 650 mμ. Rays with $\lambda > 650$ mμ can be equalized with only one — primary red — radiation; a slight admixture of violet disturbs the colour equality.

The fly does not, however, visually equalize radiations with $\lambda < 650$ mμ with red radiation — down to λ 520 mμ a suitable admixture of violet is required.

Thus, at high intensities for the eye of the fly there exist not more than two linearly independent radiations; any third radiation constitutes a linear combination.

On attaining visual equality, the wave length and relative energy both of the measured and the primary radiations were noted. The mean values of the functions for violet ($a_{1(\lambda)}$) and red ($a_{2(\lambda)}$) primary radiations obtained from 300 colour equalities on 25 male and female specimens are shown in the Table. The need for averaging the value $a_t(\lambda)$ is due to he scatter of the magnitude to be measured (from 3–5 to 20 per cent and more) which is related to the differences in the contrast sensitivity of the eye preparations (because of the individual characteristics of the flies, differences in dissection or in the state of the insect). However, after averaging the results obtained for a series of house flies we were able to find such values of $a_{t(\lambda)}$ which satisfied any specimen independent of the differences in contrast sensitivity. Thus, the equalities established in advance on the colorimeter, starting from the mean values of $a_t(\lambda)$, were always perceived by the flies both with high and low contrast sensitivity. This indicates the similarity of the spectral sensitivity of the receptors of different individuals and confirms the reliability of all the measurements.

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TABLE 1. MEAN VALUES OF FUNCTIONS $a_1(\lambda)$ AND $a_2(\lambda)$ RELATIVE TO VIOLET AND RED PRIMARY COLOURS

| λ | $a_1(\lambda)$ | $a_2(\lambda)$ | λ | $a_1(\lambda)$ | $a_2(\lambda)$ | λ | $a_1(\lambda)$ | $a_2(\lambda)$ | λ | $a_1(\lambda)$ | $a_2(\lambda)$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 365 | 0·940 | 0·0 | 480 | 1·108 | 0·0 | 550 | 0·413 | 1·060 | 650 | 0·001 | 3·500 |
| 380 | 0·420 | 0·0 | 490 | 1·140 | 0·0 | 575 | 0·100 | 3·930 | 655 | 0·0 | 1·750 |
| 390 | 0·380 | 0·0 | 510 | 1·072 | 0·0 | 590 | 0·043 | 5·740 | 680 | 0·0 | 0·300 |
| 400 | 0·340 | 0·0 | 520 | 0·925 | 0·157 | 600 | 0·013 | 6·600 | 700 | 0·0 | 0·045 |
| 420 | 0·471 | 0·0 | 540 | 0·570 | 0·630 | 620 | 0·004 | 7·000 | 710 | 0·0 | 0·004 |
| 450 | 0·820 | 0·0 | | | | 630 | 0·003 | 6·650 | | | |

Figure 3 shows two combination curves for the fly eye in percentages of the maximal value of $a(\lambda)$ for each curve. Curve *a* has a second distinct rise at the ultra-violet end of the spectrum which might have been thought to indicate the existence of a special receptor working in ultra-violet. However, such a belief is discounted by the results of the colorimetric experiment — all the spectral radiations could be matched by mixtures of two primaries taken from the short and long wave regions of the spectrum. For a more thorough check of this, the violet primary radiation was replaced by ultra-violet ($\lambda \sim 365$ mμ) but in this case all the radiations in the spectrum were indistinguishable for the fly from a mixture of two primaries and the corresponding combination curves did not differ from those shown in Fig. 3.

In all the series of experiments, two radiations indistinguishable for the fly remained indistinguishable if the energy of the first and second were reduced proportionately. The colorimeter used by us allowed us to verify this constancy (additivity) of the visual equalities to the point of an 100-fold increawe in the intensity of light in the case of one-dimensional equalities and 50-fold in the case of two-dimensional ones.

The one-dimensional colour equalities within the range 365–520 mμ were not disturbed by adaptation to rays with λ = 365 mμ. Thus, after 5 min fatiguing of the eye with powerful ultra-violet radiation (the image of the gas discharge of a mercury lamp covered with an UG-1 filter was focused on the eye) the previously matched visual equalities of radiation pairs were maintained: 380 and 435 mμ, 380 and 500 mμ, 380 and 520 mμ. This provides additional confirmation that only one receptor functions for the shortwave end of the spectrum.

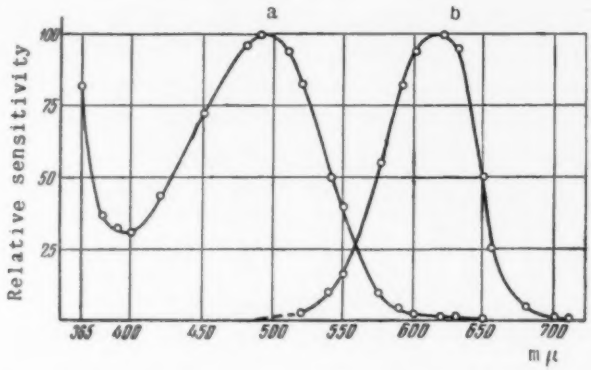


FIG. 3. Spectral sensitivity curves of eye receptors of house fly. (a) "green" receptor more sensitive to light; (b) "red" receptor functioning only in daylight luminosities. Ordinates, relative sensitivity (in percentage of maximum) for equi-energetic spectra; abscissa, λ in mμ.

The discriminatory sensitivity of the fly eye to monochromatic rays within the two-dimensional region of its visible spectrum was highest for the yellow-orange radiations. Thus, whatever the ratios of the energy of the rays with λ = 597 and 600 mμ it was impossible to ensure absence of an e.r.g. on rapid (1/300 sec) substitution of measured for primary. The fly also distinguished red rays with λ = 620 mμ and 625 mμ irrespective of the ratio of their energy. In the green, the discriminatory powers were less; the fly distinguished a ray with λ = 558 mμ from one with λ = 565 mμ but confused it with a ray with λ = 562 mμ. In the entire series of experiments the contrast sensitivity of the eye preparation was 8 per cent.

## DISCUSSION OF RESULTS

The fact that any monochromatic radiation is visually indistinguishable to the fly from a mixture of not more than two primary radiations, directly points to the presence in the eye of only two receptors with linearly-independent curves of spectral sensitivity. The house fly has two-dimensional colour vision, it is dichromatic, but with a large monochromatic region at the shortwave end of the spectrum. At the longwave end of the spectrum starting from 650 mμ, the vision of the fly is also one-dimensional since here only one receptor functions and the sensitivity of the second approximates to, or equals, zero.

The primary radiations for measuring the combination curves were selected precisely in the one-dimensional regions of the spectrum visible to the fly, i.e. violet (or ultra-violet) and red primary radiations excited only one receptor each*. Therefore, the two combination curves obtained are independent of each other and characterize the distribution of spectral sensitivity of two different receptors. They represent, as was demonstrated in discussion of the colorimetric method, the curves of spectral sensitivity of these two receptors.

At relatively low (twilight) luminosities only the "green" receptor which is more sensitive to light functions (Fig. 3 (a)). Therefore, the twilight vision of the fly is monochromatic, and the spectral sensitivity curve of the "green" receptor coincides with the scotopic visibility curve of its eye. It has two peaks: one at 490 mμ and the second, apparently, somewhere in the ultra-violet at (or near) 365 mμ. Evidence that one and not two receptors accounts for this two-peaked curve is provided by the one-dimensionality of the visual equalities in the shortwave part of the spectrum and their retention after fatiguing the eye with rays with $\lambda = 365$ mμ. The "green" receptor adapts itself to a very wide range of intensities. The spectral sensitivity of this receptor is characterized to a certain degree by the threshold two-peaked curve of sensitivity obtained by Donner [2].

The spectral sensitivity curve of the "green" receptor almost coincides with that of the parietal eye (ocellus) of the bee, as determined in the electrophysiological experiments of Goldsmith and Ruck [9], the shape of which is correlated by these workers with the presence in the bee of retinine — a component of visual purple [10]. The curve of twilight (rod) vision of the human eye with the crystalline lens removed is also similar to the sensitivity curve of the "green" receptor of the fly. It is highly probable that the similarity in the twilight vision curves of man and the fly is based on a common visual pigment, namely, rhodopsin. The common nature of the pigment is also confirmed by the similarity in the rôle of the rod apparatus of man and the "green" receptor of the fly: both are adapted to a wide range of luminosities.

The second, "red", receptor of the fly eye functions only at relatively high (daylight) luminosities (Fig. 3 (b)). Therefore, we were not able to discover with our colorimeter (because of the low intensity of the monochromatic blue and blue-green rays) how its sensitivity falls off in the shortwave region of the spectrum starting from $\lambda = 520$ mμ. But it is quite probable that in natural daylight luminosities the "red" receptor will be sensitive also to the blue-green rays down to $\lambda = 490$ mμ. The "red" receptor has one maximum at $\sim 620$ mμ on both sides of which the sensitivity falls quite sharply.

Thus, the two-dimensional region of the spectrum to which both receptors are sensitive in fact encompasses radiations with λ from 490 to 650 mμ. The discriminatory

* The energy maximum of the red primary radiation, bearing in mind the character of the energy distribution in the light passing through the red primary slit, was reached for $\lambda = 650$–$660$ mμ. But the sensitivity of the "green" receptor to this portion of the spectrum was very low (for $\lambda = 650$ mμ it was 0·1 per cent of the maximum at 490 mμ). Consequently, it may be considered that the red primary radiation in practice excited only the "red" receptors.

sensitivity to pectral rays especially in the middle portion of the two-dimensional region of the spectrum, is quite high; the fly distinguishes monochromatic colours with a difference in $\lambda$ of 3–7 m$\mu$.

As a whole, the properties of colour vision of the fly can be characterized in the terms used by man to describe his sensations of colour. For the fly colours which would be absent from the spectrum do not exist. All mixed colours containing either all the spectral rays (white and grey colours) or the rays from the opposite ends of the spectrum (purple) with a certain ratio of luminosities are indistinguishable from green, yellow, orange and near red*. For example, equi-energetic white (and also similar greys) and a multiplicity of purple colours and a certain spec ral green with $\lambda < 560$ m$\mu$ are visually identical for the fly, since for a certain matching of brightness, they all uniformly excite both receptors. The colours blue, violet and also ultra-violet radiations for a certain combination of luminosities, cannot be discriminated by the fly but are distinguishable from all hues of blue-green, green, yellow, etc., to the far red colours.

Knowing the spectral sensitivity curves of the receptors it is easy to estimate the physical characteristics of the particular colours not distinguished by the fly, and, conversely, from the physical characteristics of the given radiations one can tell in advance whether or not they will be distinguishable to the fly eye.

Among the multiplicity of insect species only in three; the honeybee [11], the dragonfly *Libellula* [7] and the fly *Calliphora* [4, 12], have the properties of colour vision been studied in any detail, although the spectral sensitivity of the receptors are known only for the dragonfly. Colour vision in the house fly as compared with these insects is closest to that of *Calliphora* judging from the colours described by Autrum [4] as distinguishable and indistinguishable by *Calliphora* and certain observations of Schneider [12] on its behaviour in an optomotor drum. The peaks of the curve of spectral sensitivity of *Calliphora* [4, 5] in number and position in the spectrum, roughly coincide with the maxima of sensitivity of both receptors of the house fly. However, the peak of this curve in the ultra-violet region of the spectrum is probably related not to the existence in the eye of *Calliphora* of a special "ultra-violet" receptor, as postulated by Walther, but to the presence of two maxima of sensitivity for one and the same receptor.

The "red" receptor of the house fly is similar to the "red" receptor of the lower part of the dragonfly eye, but their "green" receptors are somewhat differen : the spectral sensitivity curve of the "green" receptor of the dragonfly is shifted towards the longer wave radiations and apparently does not show a sharp rise in the ultra-violet. In addition, the upper part of the dragonfly eye has one-dimensional vision in which a different "violet" receptor operates [7].

The system of colour vision of the honeybee is more sophisticated. Bees are, apparently, trichromates [11] although this cannot be stated for certain since no

* This explains the failure to train flies to discriminate green from grey [3]. The samples of green and grey paper used, were, in these conditions of illumination, similar visually in colour for the fly.

successive colorimetric experiments have been carried out on them. Therefore, the properties of colour vision of bees, despite the 50 year history of study from Forel [13] to Daumer [11], even now are not as fully known as they are for the house fly or dragonfly.

## SUMMARY

Using a substitution colorimeter [7] we found physically different radiations visually indistinguishable to the house fly and obtained "colour combination curves". The absence of an e.r.g. on rapid replacement of illumination of the eye with monochromatic radiation by a strictly defined mixture of primary radiations (and vice versa) was evidence of the visual identity for the fly of the given pair of radiations.

Any spectral colour is indistinguishable for the fly from a mixture of not more than two primary colours, which points to the existence in the eye of only two light receptors with linearly-independent spectral sensitivity curves. The longwave "red" receptor is maximally sensitive to $\lambda \sim 620$ mμ, and the shortwave "green receptor has two peaks of sensitivity: at 490 mμ and $\sim 365$ mμ in the ultra-violet (Fig. 3). The dichromatic region of the spectrum visible to the fly to which both receptors are sensitive is confined to $\lambda = 490$–650 mμ. With twilight illumination the vision of the fly is monochromatic since at low luminosities only the "green" receptor functions. The article gives an analysis of the colour discrimination properties of the vision of the fly.

*Translated by* A. Crozy

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# PARTICIPATION OF ELECTRIC POTENTIAL DIFFERENCES IN THE TRANSLOCATION OF PHOSPHORUS ($^{32}P$) IN THE ROOTS OF THE ONION*

N. A. GORLANOV

The Lobachevskii State University, Gor'kii

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VOL
5
196

THE possibility that electric potential differences (p.d.) may be involved in the intake and transpart of substances in plants has been repeatedly mentioned in the literature [1–5]. In particular, Lund has suggested that differences in the surface electrical potentials, constantly maintained by the plant organism, may perform a function in the basic metabolic processes, including the selective transport of ions [2].

This idea was experimentally confirmed in the work of Opritov who used, in our view, an interesting technique for studying the transport of substances in plant tissues, and the simultaneous change in the p.d. by shunting the tissues and by local treatment with $CdCl_2$ [4]. However, the author himself points out that his experimental results are of a tentative character and require special checking.

The aim of the present article is to present some additional information suggesting the participation potential differences in the transport of $^{32}P$ through the plant.

The experiments were conducted on roots of the onion. In this work we were concerned with a study of the transport of $^{32}P$ from the plant leaves to the roots against a background of changed bioelectrical potentials of the roots. We varied the p.d. of the onion roots by immersing them in a conducting liquid, i.e. by shunting, which in a short experiment is a very mild factor with little effect on the other vital functions of the organism [2, 6–8].

The design of the experiments was as follows. The young onion crop of the Novolikeyevskii variety was grown in quarter-strength Knop's medium (pH 6·7). After 14–15 days the plant roots (9–10 cm long) were carefully washed consecutively with tap, distilled and double distilled water. The whole bundle of roots of each plant was divided into three equal groups, underdeveloped roots were excluded from the experiment. Each group of roots was shunted by a conductor of different electrical conductivity.

The first group of roots was shunted with double distilled water with a specific electrical conductivity of $\varkappa = 0{\cdot}0000081\ \Omega^{-1}\ cm^{-1}$, the second wi h tap water with $\varkappa = 0{\cdot}000554\ \Omega^{-1}\ cm^{-1}$ and the third group with 0·01 N NaCl with $\varkappa = 0{\cdot}001152\ \Omega^{-1}$

* Biofizika **5**: No. 3, 304–307, 1960.

$cm^{-1}$. Shunting was carried out by dipping the roots into vessels containing these solutions. Then diluted Knop's medium containing $NaH_2$ $^{32}PO^4$ was added to the plant leaves at the rate of 0·2 μc per plant. The tips of the roots (5 cm) were cut off from the plants and fixed 7–12 hr after the time of introducing. In the ground up material of each group the $^{32}P$ activity was estimated with a B-2 unit and a MST-17 tube.

The results of the experiments are listed in Table 1. They clearly show that dipping onion roots into such liquid conductors as tap water and 0·01 N NaCl causes a considerable reduction in the passage of $^{32}P$ from the plant leaves to the tips of the roots as compared with the group in which the roots were immersed in double distilled water, the degree of reduction in transport of $^{32}P$ from the leaves to the root tips being a function of the electrical conductivity of the shunting solution.

VOL.
5
1960

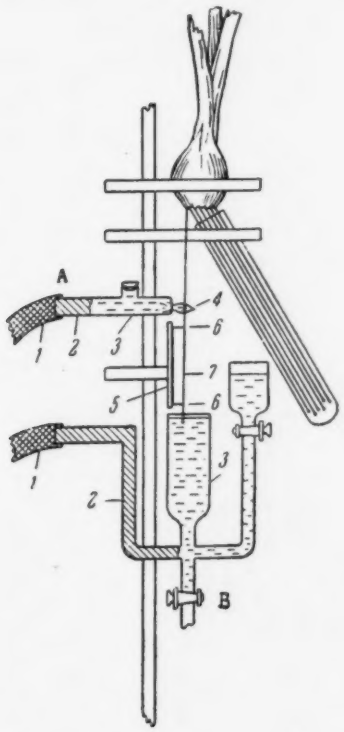


FIG. 1. Diagram of contact apparatus for determining p.d. of onion roots on shunting. (A) attachment for upper contact of electrode with object; (B) attachment for lower contact of electrode with object; (1) intermediate link (agar + KCl); (2) agar + water; (3) water; (4) camel hair brush; (5) moving lever for securing shunt; (6) shunt; (7) root.

However the question arises as to how the value of the p.d. is related to the electrical conductivity of the conducting liquids we used for shunting the p.d. To this end, measurements were made of the p.d. of the onion roots on shunting. The p.d.

was measured by an electrometer value [9]. We employed calomel electrodes which were connected through an intermediate link (agar + KCl) to the contact apparatus (see Fig. 1). Contact with the object was achieved by dipping the root 3 mm in water and by applying a camel hair brush, moistened with water, to a section of the root

TABLE 1. TRANSPORT OF $^{32}P$ IN ONION ROOTS WITH SHUNTING OF THEIR POTENTIAL DIFFERENCE. (ACTIVITY IN COUNTS PER MIN PER MG OF DRIED SUBSTANCE)

| Shunt | Fixation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | After 7 hr | | | | After 12 hr | | | |
| | Plants | | | | | | | |
| | First | Second | Third | Fourth | First | Second | Third | Fourth |
| Double distilled water | 776±6 | 660±5 | 653±4 | 634±4 | 1190±10 | 883±8 | 968±8 | 879±7 |
| Tap water | 506±4 | 463±3 | 490±4 | 398±3 | 687±6 | 615±5 | 790±6 | 612±5 |
| 0·01 N NaCl | 407±3 | 236±2 | 302±3 | 281±2 | 541±5 | 196±2 | 436±3 | 501±4 |

40 mm from its tip. As shunts, we used sheets of filter paper shaped like a clamp and moistened with the shunting fluid. The shunt was placed on a movable lever made from an insulator. A 30 mm length of root was shunted, the shunt touched the root only at two points which were 3–4 mm away from the electrode contacts. The area of contact of shunt and root was equal to the thickness of the filter paper which reduced to a minimum the effect of the solutions on the object and the effect of contact. The

TABLE 2. SHUNTING EFFECT OF A CONDUCTING LIQUID ON THE POTENTIAL DIFFERENCE OF ONION ROOTS (mV)

| Plant No. | Double distilled water | | | Tap water | | | 0·01 N NaCl | | |
|---|---|---|---|---|---|---|---|---|---|
| | Before shunting | During shunting | After shunting | Before shunting | During shunting | After shunting | Before shunting | During shunting | After shunting |
| 1 | 60 | 50 | 58 | 56 | 30 | 55 | 60 | 20 | 60 |
| 2 | 58 | 50 | 58 | 68 | 32 | 66 | 66 | 32 | 65 |
| 3 | 48 | 46 | 47 | 56 | 32 | 57 | 71 | 20 | 70 |
| 4 | 64 | 50 | 64 | 50 | 30 | 50 | 59 | 24 | 60 |
| 5 | 70 | 54 | 69 | 64 | 34 | 62 | 61 | 25 | 62 |
| 6 | 72 | 58 | 71 | 59 | 32 | 60 | 56 | 23 | 55 |
| 7 | 58 | 48 | 59 | 57 | 33 | 57 | 53 | 29 | 50 |
| 8 | 53 | 49 | 54 | 62 | 34 | 59 | 62 | 20 | 63 |
| 9 | 57 | 47 | 57 | 58 | 31 | 60 | 62 | 23 | 61 |
| 10 | 63 | 53 | 61 | 60 | 32 | 61 | 60 | 24 | 59 |
| Average | 60·3 | 50·5 | 59·8 | 59·0 | 32·0 | 58·7 | 61·0 | 24·0 | 60·5 |
| Ratio of p.d. value before shunting to that on application of shunt | | 1·2 | | | 1·8 | | | 2·5 | |

p.d. of the roots was measured before shunting, with the shunt applied and after removal of the shunt. The time of shunting was 3–5 min. The whole process took not more than 10–12 min. Such a method of shunting is a convenient expedient and makes it possible to obtain precise information on the variation in the values of the p.d. Each group involved not less than ten roots of the same plant.

Similar results were obtained in both experiments, the results for one of them being shown in Table 2.

Table 2 shows that on application of the shunt to the root the p.d. was diminished in all groups, but the degree of reduction varied. Thus, with a shunt moistened with double distilled water, the p.d. fell by a factor of 1·2 as compared with the values for the p.d. before shunting; with a shunt moistened with tap wate , by a factor of 1·8, and with a solution of NaCl, 2·5 times, i.e. the degree of reduction of the p.d. of the roots is a function of the electrical conductivity of the shunting liquid. After removing the shunt the p.d. was rapidly restored to the former level. This shows that the change in p.d. values on shunting is obviously not a result of gross disturbance in the vital functions of the root, but solely a result of the leakage of electric potentials. However, the times and methods of shunting in these experiments were not identical with those in previous experiments on transport of $^{32}P$. In order to satisfy ourselves that shunting of the p.d. by dipping the roots into solutions only slightly affects other functions of the roots, the p.d. values of the roots were measured after they had been kept for 12 hr in the above-mentioned solutions and rinsed with distilled water. No fundamental difference was found between the original p.d. values and those of all three group after the experiment (see Table 3).

TABLE 3. VALUES OF POTENTIAL DIFFERENCE IN ONION ROOTS AFTER KEEPING FOR TWELVE HOURS IN SHUNTING SOLUTION (mV)

| Before shunting | After removal of shunt | | |
|---|---|---|---|
| | Double distilled water | Tap water | NaCl solution |
| 53 | 50 | 51 | 59 |

Hence, leakage of electrical potentials leads to inhibition of $^{32}P$ transport; the degree of reduction in the p.d. and the degree of inhibition of $^{32}P$ transport are a function of the electrical conductivity of the shunt. This indicates that the e.m.f. of the cells and tissues plays a definite part in the transport of substances in plants.

Obviously, the electric p.d. may take part in the transport of substances in plants in various ways, as pointed out in the literature [2, 9–13]. Elucidation of the particular modes of this participation is undoubtedly of great theoretical interest and will enable us to gain a wider understanding of the physiological role of potential differences in plants.

*Translated by* A. CROZY

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* There is a translation into English of this work.


VOL
5
196

# STUDY OF THE OPTICAL PROPERTIES OF LEAVES IN RELATION TO THE ANGLE OF INCIDENCE OF LIGHT*

S. V. TAGEYEVA and A. B. BRANDT

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

(*Received* 14 *July* 1959)

ILLUMINATION of plant leaves by solar rays during the day occurs at a constantly changing angle of incidence. Therefore, it is necessary to know to what extent the optical properties, especially absorption of light by the leaf, may depend on change in the angle of incidence of illumination.

The leaf is a heterogeneous system owing to the presence in it of elements with very complex structural organization, and with variable optical density. The rays of light on entering such a heterogeneous medium undergo repeated refractions and reflexions at different angles resulting in considerable scatter of light and secondary absorption within the leaf.

In the literature there are a number of papers in which the problem of the scattering powers of leaves are considered on the basis of the nature of the characteristic curve of the reflected and transmitted light at a constant, unchanging angle of incidence [1–3].

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It must be emphasized that for biologists, knowledge of the indicatrix of scatter of reflected light and light transmitted through the leaf is not of great interest, since only the light absorbed by the leaf is capable of producing all the physiological processes in living plants. Therefore, we concluded that it was necessary to measure the coefficients of absorption, reflexion and transmission by the leaf of light of variable spectral composition, in relation to the different angles of incidence of the irradiating light found in natural conditions.

The measurements were made with a universal apparatus for studying the optical properties of plant leaves [4] in white light from a 500 W cineprojection lamp, and in monochromatic light obtained by interference light filters at $\lambda = 404$, 463, 545 and 656 mμ.

For the investigation, quite thick shiny leaves were used — *Ficus elastica* — thinner leaves with a shining surface, *Hibiscus rosa* sinensis and a leaf with very bright lamina — *Lactuca sativa*. In addition, it was of interest to investigate the leaves of *Coleus* which have anthocyanins in the cells of the upper epidermis and a velvety, matt surface.

In order to find out the rôle of the particular structural leaf elements in the absorption and scatter of light, we used in the experiment — together with normal leaves — firstly, leaves infiltrated with water i.e. with considerably reduced heterogeneity but with he pigment apparatus retained and secondly, yellow autumn leaves with unchanged heterogeneity and disturbed pigment composition.

Figures 1–4 show, in polar co-ordinates, the results of measurements on normal leaves. These give a vivid idea of the change in the coefficients of reflexion, transmission and absorption in relation to the angle of incidence of the light.

The pigment content in the leaves, investigated by the method of Godnev, is indicated in Table 1.

TABLE 1

| Plant | Pigment concentration | | | |
|---|---|---|---|---|
| | mg/100 cm² | | mg/g | |
| | Chlorophyll | Carotinoids | Chlorophyll | Carotinoids |
| *Ficus elastica* | 9·27 | 2·21 | 2·82 | 0·618 |
| *Hibiscus rosa* sinensis | 3·67 | 1·38 | 2·520 | 0·96 |
| *Lactuca sativa* | 1·35 | 0·52 | 1·44 | 0·57 |

The figures make it quite clear that at an angle of incidence of 0° reflexion was minimal; with an increase in the angle of incidence the reflexion was considerably increased. A similar pattern was observed both for the thin, matt (Fig. 1) and dense (Fig. 2) and thick (Fig. 3) leaves wit a shiny surface. A different picture was observed for the coefficient of transmission of light by the leaves. With vertical illumination maximum transmission was noted becoming minimal on i lumination at an angle of 75°.

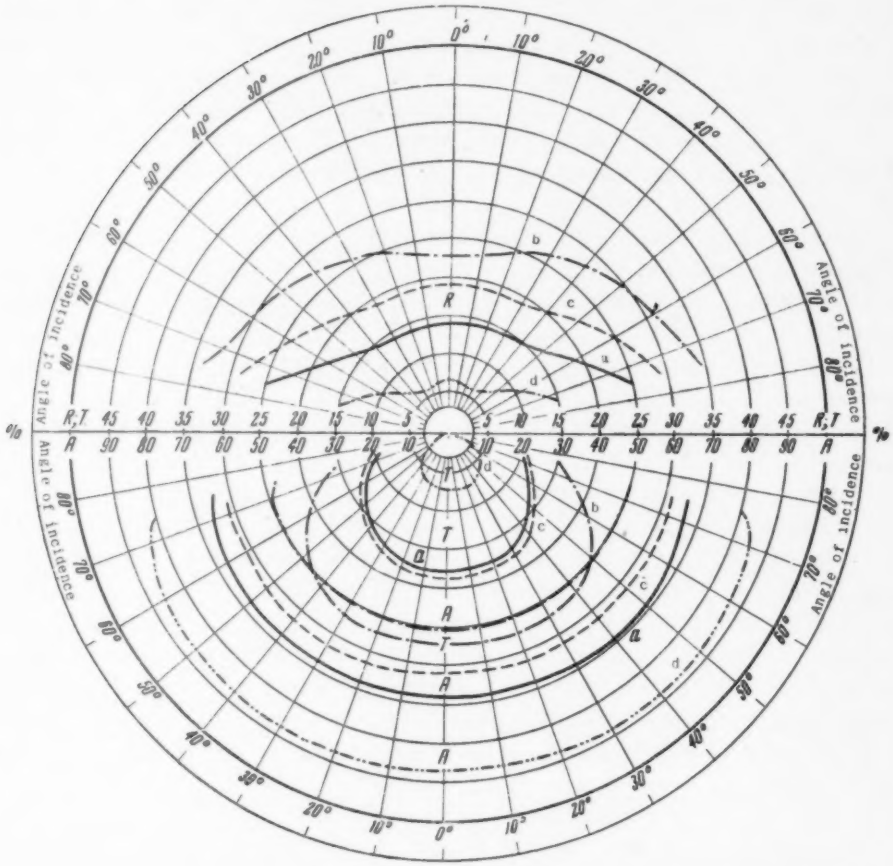


FIG. 1. Coefficients of reflexion (R), transmission (T) and absorption (A) of light by leaf of *Lactuca sativa* in relation to change in angle of incidence of light of various spectral regions. (a) white light; (b) $\lambda = 545$ mμ; (c) $\lambda = 656$ mμ; (d) $\lambda = 463$ mμ. For ease of inspection, the curves of the reflexion coefficient are given above the horizontal axis and the curves of the transmission and absorption coefficients beneath it. Scale of curves of reflexion (R) and transmission (T) coefficients is given above horizontal axis and is double that for the curves of absorption (A) coefficient which is given beneath the horizontal axis.

Precisely because for an increase in the reflexion coefficient a decrease in he coefficient of transmission of light, for angles of incidence from 0 to 55°, is observed the coefficient of absorption of different wave lengths of light by the leaf remains practically constant. Only at angles of incidence greater than 60° was the coefficient of absorption slightly reduced.

The experiments with *Coleus* (Fig. 4) show that owing to the presence in the leaf of an additional pigment — anthocyanin — there is a considerable increase in the coefficient of absorption and a corresponding decrease in the coefficients of transmission

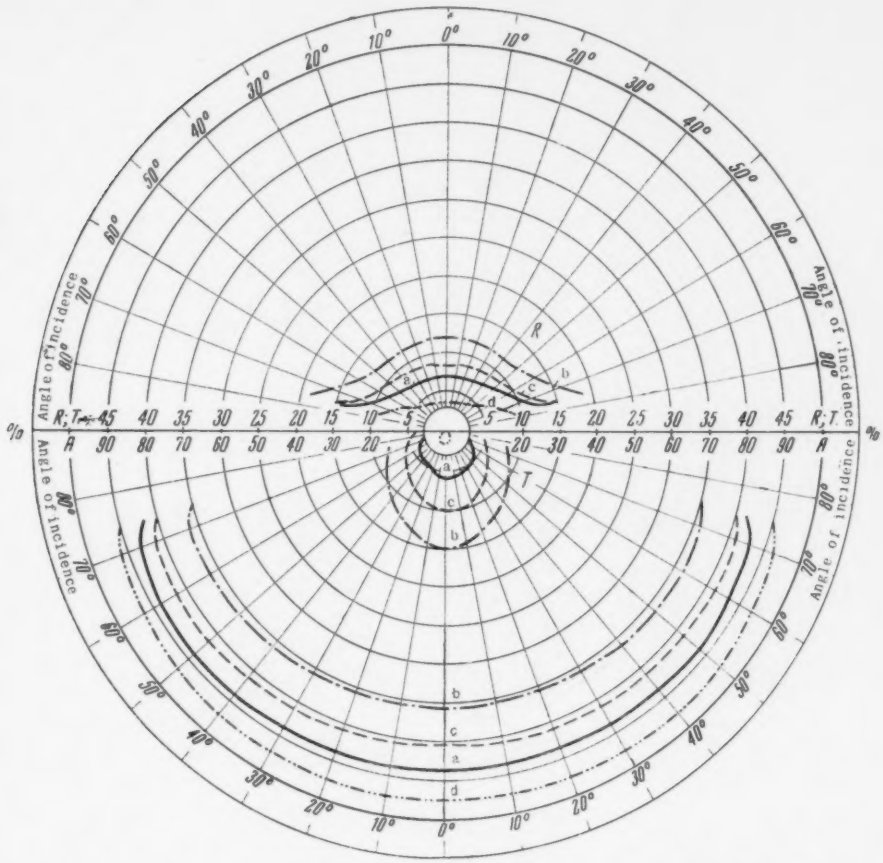


FIG. 2. Coefficients of reflexion (R), transmission (T) and absorption (A) of light by leaf of *Hibiscus rosa* sinensis with change in angle of incidence of light of various spectral regions (a) white light; (b) $\lambda = 545$; (c) $\lambda = 656$; (d) $\lambda = 463$ mμ.

and reflexion, but the character of the curves of the optical parameters in relation to the angle of incidence of light remain the same as in normal green leaves.

We consider that the experimental results reported here prevent our concluding that leaves have ideal scattering powers since there are large variations in the coefficients of transmission and reflexion of light for the leaf with the angle of incidence of light. A medium can only be termed ideally scattering, if transmission and reflexion of light in it are governed by Lambert's law and the brightness is uniform in all directions irrespective of the direction of the radiation incident on the medium [5].

At the start of this article, we pointed out that the optical inhomogeneity of the structure of the tissues of the leaf determines its complex optical properties as a turbid medium with high powers of scatter. Therefore, it may be assumed that with a decrease in this inhomogeneity (for example, attenuating it by expelling air from the intercellular

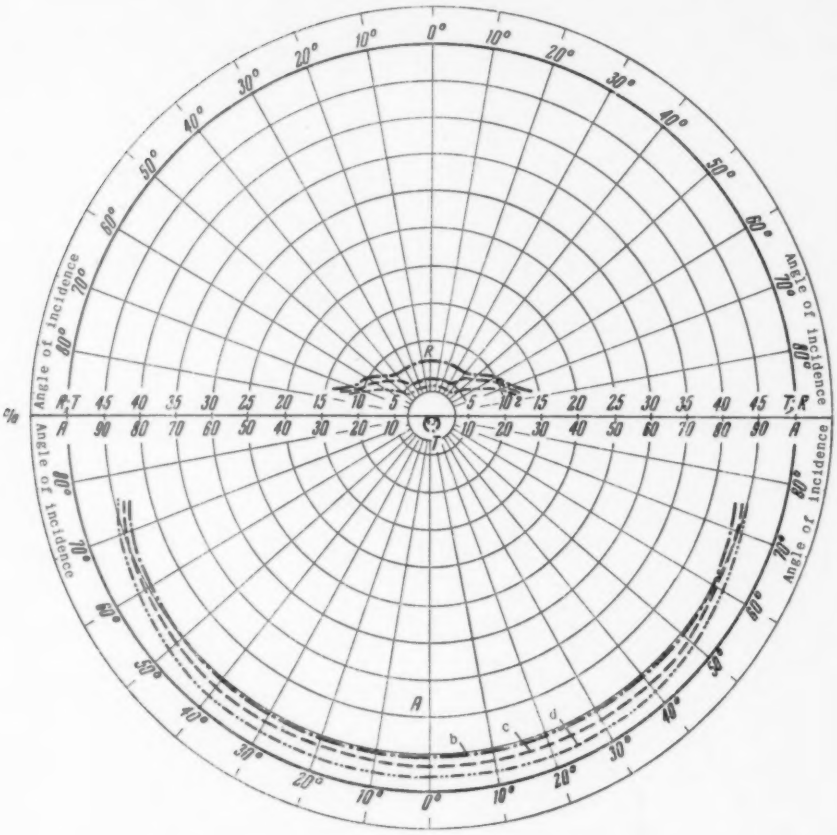


FIG. 3. Coefficients of reflexion (R), transmission (T) and absorption (A) of light by leaf of *Ficus elastica* with change in angle of incidence of light of various spectral regions. (b) $\lambda = 545$; (c) $\lambda = 656$; (d) $\lambda = 463$ mμ.

spaces and replacing the gaseous phase by a liquid phase — water) we should lessen the scattering powers of leaf issues and change the optical parameters of the leaf.

Accordingly, we investigated the optical properties of leaves completely infiltrated with water. The results are given in Table 2 and suggest the conclusion that expulsion of air by water from the intercellular spaces does, in fact, considerably reduce scatter of light within the leaf, and primarily reduces the coefficient of reflexion of light from the surface of the leaf, especially in the thin leaf of *Lactuca* (Fig. 5). The coefficient of absorption of light in the *Lactuca* leaf also sharply fell. Filling of the intercellular spaces with water sharply reduced the internal multiple refractions and reflexion of light and thereby lessened the possibility of repeat absorption of light within the leaf, while, on the other hand, it contributed to the unhindered passage of light through a more homogenous medium with a low concentration of pigments actively absorbing

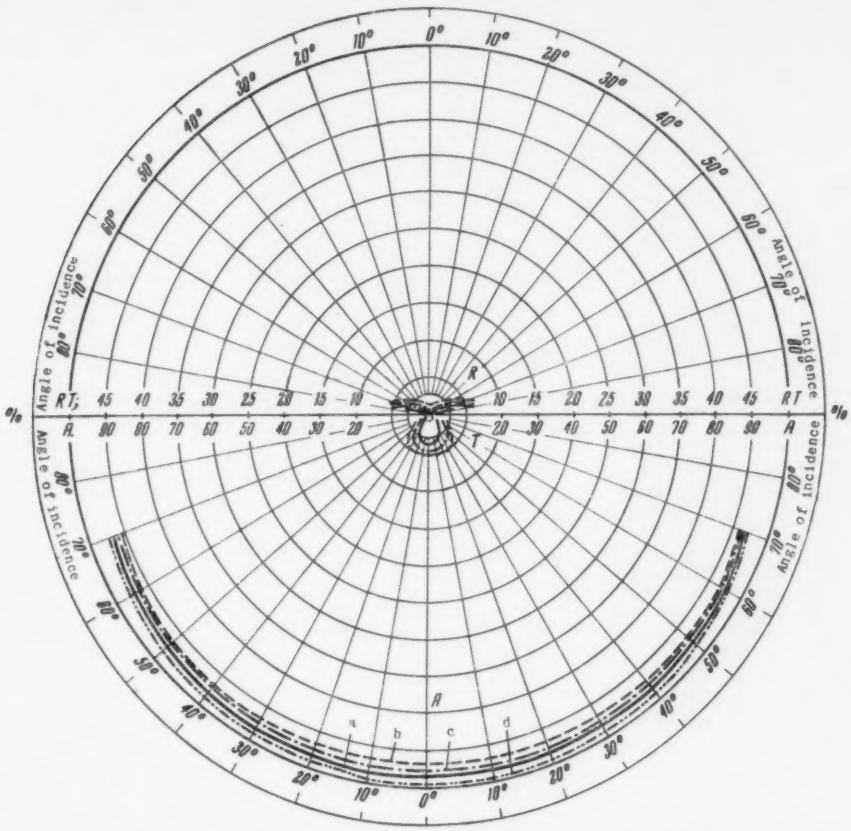


FIG. 4. Coefficients of reflexion (R), transmission (T) and absorption (A) of light by leaf of *Coleus* with change in angle of incidence of light of various spectral regions. (a) white light; (b) $\lambda = 545$; (c) $\lambda = 656$; (d) $\lambda = 463$ mμ.

TABLE 2

| Plant species | Wavelength of light, (mμ) | Normal leaf | | | Infiltrated leaf | | | | | Pigments mg 100 cm² | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Absorption | Reflection | Transmission | Absorption | Percentage of controls | Reflection | Percentage of controls | Transmission | Percentage of controls | Chlorophyll | Carotins |
| *Lactuca sativa* | White | 77 | 12 | 11 | 47 | 60 | 6 | 54 | 47 | 454 | | |
| | Red, = 656 mμ | 58 | 16 | 26 | 38 | 66 | 7 | 46 | 55 | 210 | 1·35 | 0·52 |
| | Green, = 545 mμ | 51 | 23 | 26 | 21 | 40 | 9 | 42 | 70 | 266 | | |
| | Blue, = 463 mμ | 86 | 7 | 7 | 71 | 82 | 5 | 73 | 24 | 360 | | |
| *Hibiscus rosa sinensis* | White | 87 | 7 | 6 | 82 | 94 | 6 | 90 | 12 | 210 | | |
| | Red, = 656 mμ | 82 | 8 | 10 | 72 | 88 | 8 | 93 | 20 | 202 | 3·67 | 1·38 |
| | Green, = 545 mμ | 73 | 12 | 15 | 61 | 84 | 9 | 74 | 30 | 196 | | |
| | Blue, = 463 mμ | 95 | 4 | 1 | 94 | 99 | 4 | 97 | 2 | 154 | | |

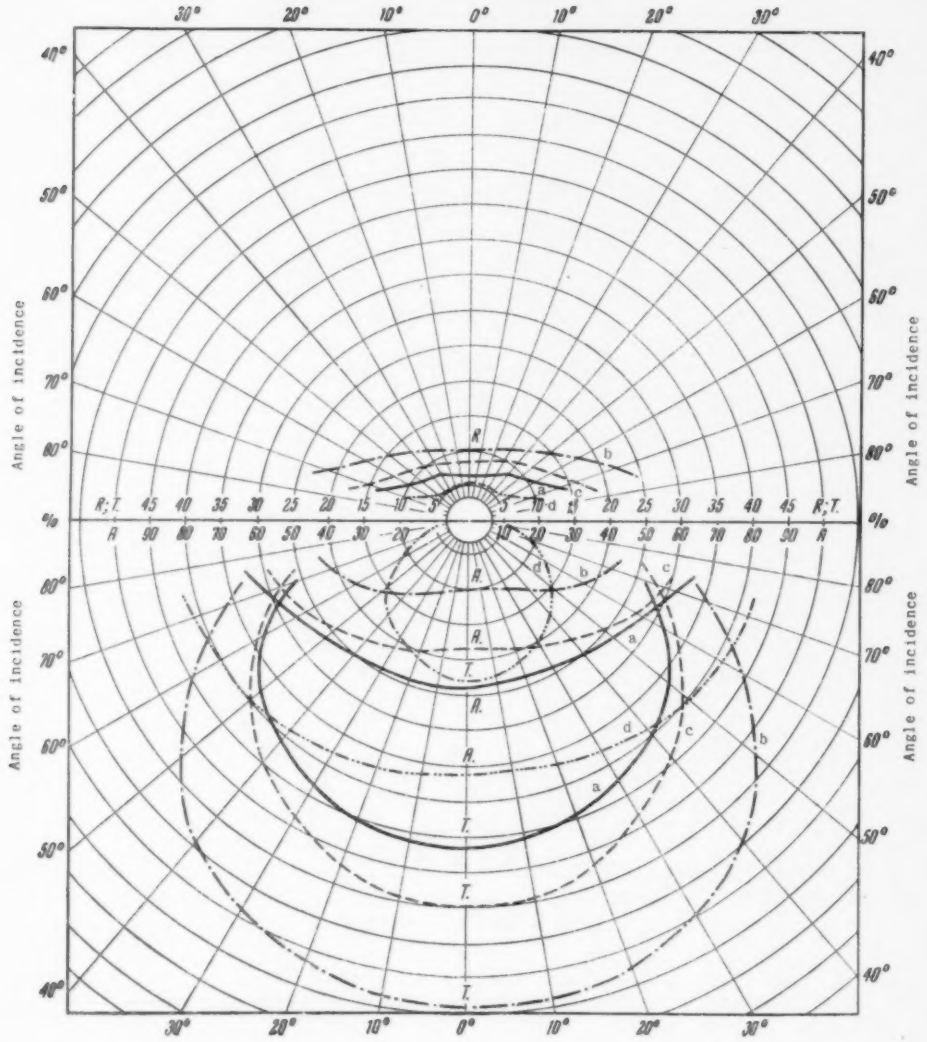


FIG. 5. Coefficients of reflexion (R), transmission (T) and absorption (A) of light by water-infiltrated leaf of *Lactuca sativa* with change in angle of incidence of light of various spectral regions. (a) white ilght; (b) $\lambda = 545$; (c) $\lambda = 656$; (d) $\lambda = 463$ mμ.

the light rays. In fact, the coefficient of transmission of light by *Lactuca* leaves rose 2–3 times as compared with the controls.

Much less marked was the change in the optical paramete s for the denser and pigment-rich infiltrated leaf of *Hibiscus rosa* sinensis (Fig. 6) as compared with the controls (Fig. 2). Thus, in green light (545 mμ), poorly absorbed by leaf pigments, on infiltration of the leaf, light absorption in *Lactuca* fell 2·5 times and *Hibiscus rosa* sinensis 1·2 times. In the strongly absorbed blue light (463 mμ) absorption was practi-

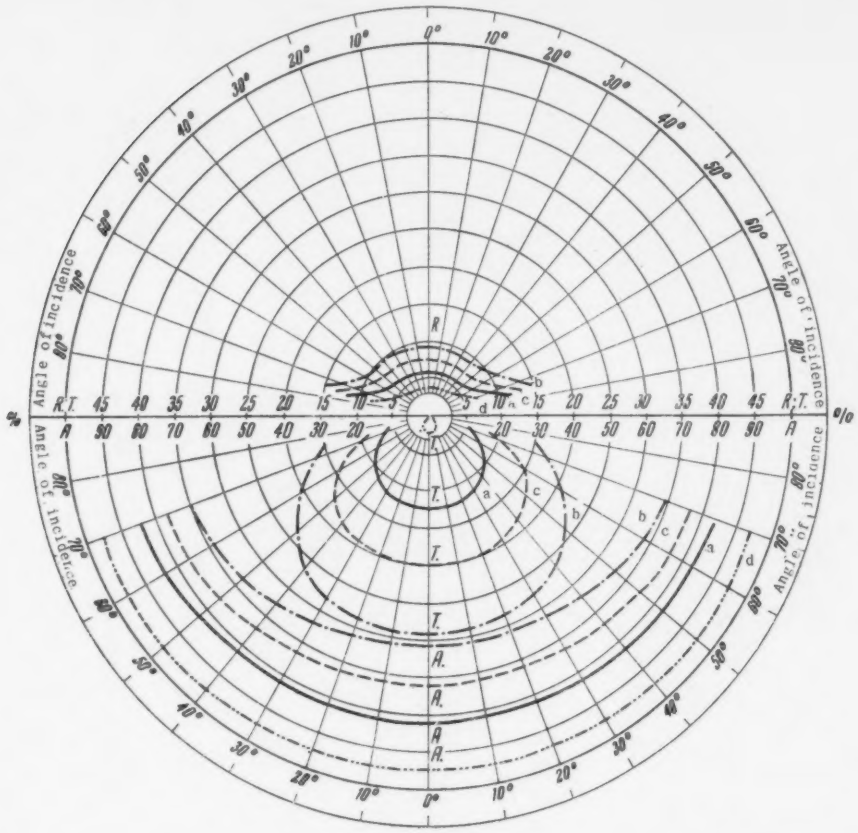


FIG. 6. Coefficients of reflexion (R), transmission (T) and absorption (A) of light by water-infiltrated leaf of *Hibiscus rosa* sinensis with change in angle of incidence of light of various spectral regions. (a) white light; (b) $\lambda = 545$; (c) $\lambda = 656$; (d) $\lambda = 463$ mμ.

cally unchanged both in *Lactuca* and especially in *Hibiscus rosa* sinensis. These results are in good agreement with the findings of Seybold [6] and also Moss and Loomis [7].

The results of our study of the optical properties of the leaf on infiltration with water allow a number of inferences to be drawn on the importance of the system of intercellular spaces in the mesophyll of the leaf. The air-filled spaces serve not only as a system whereby gaseous exchange takes place in the leaf but also contribute to the greater scatter of the light incident on the leaf and so may protect the chlorophyll from destruction by direct solar rays. In addition, this internal scatter of light may ensure its more fuller utilization on repeat internal reflexions, especially in pigment-deficient leaves (*Lactuca*) by increasing the light absorption coefficient in the poorly absorbed green region and to a lesser degree in the red region of the spectrum.

In the blue region, best absorbed by the leaf, in which two pigment systems are involved in the absorption (chlorophyll and carotinoids) infiltration by water had

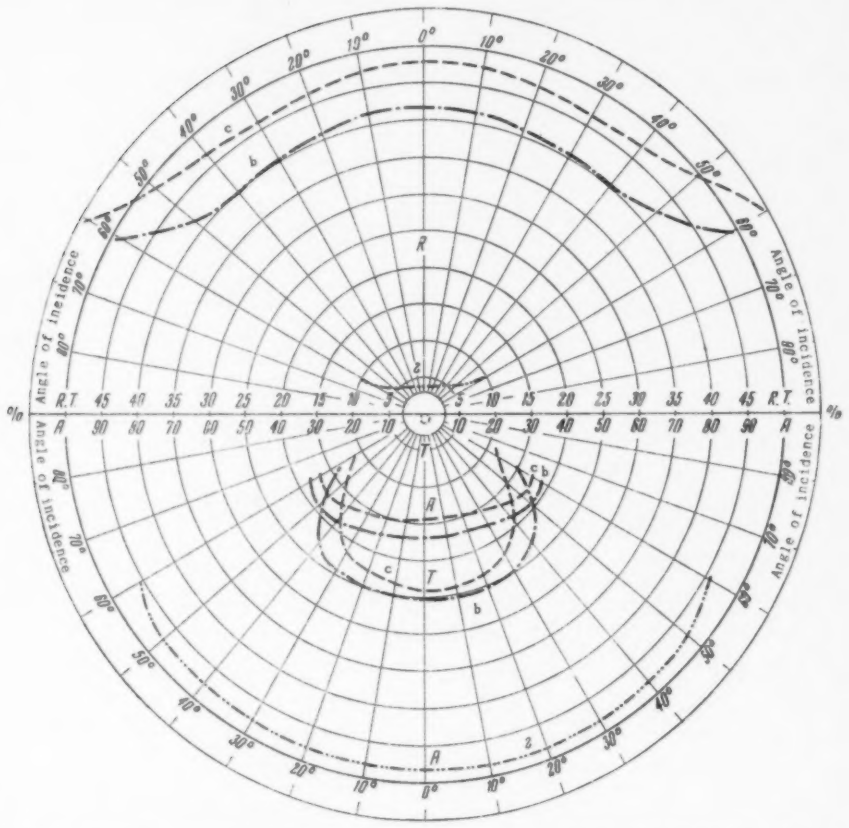


FIG. 7. Coefficients of reflexion (R), transmission (T) and absorption (A) of light by the yellowed leaf of *Ficus elastica* with change in angle of incidence of light of various spectral regions. (b) $\lambda = 545$; (c) $\lambda = 656$; (d) $\lambda = 463$ mμ.

no effect on the absorption of light by the leaf (*Hibiscus rosa* sinensis) or affected it to an insignificant degree (*Lactuca*). One cannot fail to agree with Rabinovich [3] that although the geometrical optics of the leaf are of great importance in defining its optical properties, prime importance attaches to the pigment systems actively absorbing the radiant energy.

Still more convincing confirmation of this thesis is provided by the results given below on the determination of the optical parameters of yellowed leaves. In the yellow leaf of *Ficus* (Fig. 7) the reflexion coefficient rose for $\lambda = 545$ and 656 mμ and also the transmission coefficient increased. However, at $\lambda = 463$ mμ very slight changes were observed. As for the absorption coefficient, it sharply fell for $\lambda = 545$ and 656 mμ and remained almost unchanged for $\lambda = 463$ mμ.

It is known that carotinoids remain unchanged for a long time after yellowing of leaves and destruction of chlorophyll and therefore play a major part in the absorption

of blue light. At the same time destruction of chlorophyll leads to a sharp change in the optical indices in green and red light. But it is interesting that the breakdown products of chlorophyll still remain able, to some extent, to absorb the rays at $\lambda =$ 656 mμ. It is particularly important to note that with pronounced qualitative changes in the optical parameters in yellow lesves, the course of the curves of reflexion, transmission and absorption coefficients in relation to the angle of incidence of light remain the same as in normal leaves.

We shall now consider how the optical properties of leaves infiltrated with water change with the angle of incidence of the irradiating light (see Fig. 5 and 6). Reflexion of light is lessened because of the reduction in the back reflexion at the boundary of the two phases (cell membrane, water). However, the general regularity of the course of the curve of the reflexion coefficient with change in the angle of incidence of the irradiating light remains the same as in the non-infiltrated leaf, i.e. with an increase in the angle of incidence the reflexion coefficient rises.

The transmission coefficient on infiltration of the leaf rises appreciably but as in the normal leaf with an increase in the angle of incidence the transmission coefficient decreases.

The greatest deviations from normal were observed on the absorption of light by the infiltrated, thin leaves of *Lactuca* (Fig. 5). With an increase in the angle of incidence of light on the leaf the absorption coefficient rose substantially and the degree of this increase varied with the spectral composition of the irradiating light. Thus for rays $\lambda = 545$ mμ the absorption coefficient rose from 21 to 44 per cent with change in the angle of incidence from 0 to 75° and for the rays $\lambda = 463$ mμ the rise in the absorption coefficient was only from 71 to 84 per cent.

On infiltration of the leaf of *Hibiscus rosa* sinensis with water (Fig. 6), a leaf rich in pigments and with an epidermis having a dense cuticular layer, in strongly absorbed blue rays change in the angle of incidence of the irradiating light did not cause any changes in the optical properties of the leaf. With regard to the poorly absorbed green light, in the same way as for *Lactuca*, its absorption by the leaf rose with increase in the angle of incidence.

The rise in the coefficient of absorption of light by the leaf with increase in the angle of incidence of the irradiating light in the infiltrated leaves, may be attributed to the fact that as a result of infiltration the heterogeneity of the leaf is lessened. Consequently, the leaf to a certain degree starts to come under the laws of geometrical optics so that at a large angle of incidence of light on the leaf, the path travelled by the light in the leaf increases with a proportional increase in absorption.

But if we compare the findings on the optical parameters of the infiltrated leaves with the controls, we see that the absolute values of the absorption coefficients in infiltrated leaves at all angles of incidence of light are less than in the controls.

Because of the fact that at mean latitudes around noon during summer, the maximal altitude of the sun above the horizon is 50–55°, and the surface subtended perpendicular to the incident rays (angle of incidence 0°) is subject to maximum radiation,

then the inclined disposition of leaf lamina to the horizon in many mesophytic plants becomes understandable. For the greater part of the bright period of the day, the leaves are in the most favourable conditions (in relation to the direction of the incident rays) to have the highest absorption coefficient of light for the leaf.

Thus, thanks to the inclined position of leaves, plants adjust themselves to the maximum absorption of solar radiation.

## SUMMARY

(1) For a correct understanding of a whole number of physiological processes occurring in plants and changing with the altitude of the sun above the horizon biologists must know the variation in the coefficient of absorption of light by the leaf for given angles of incidence of the radiating light.

(2) The coefficient of absorption of light by the leaves of different species with change in the angle of incidence through 0–60° remains practically unaltered, only with increase in the angle of incidence of light above 60° does the absorption coefficient fall slightly.

(3) The coefficient of reflexion of light by the leaf sharply rises with an increase in the angle of incidence, the transmission coefficient, in contrast, falls sharply.

(4) The curves of the coefficients of absorption of light of differing wavelengths ($\lambda$ = 433, 545 and 656 m$\mu$) are of a uniform character and do not depend on the quality of the irradiating light. This also holds good for the spectral coefficients of reflexion and transmission.

(5) Infiltration of leaves increases the transmission coefficient for rays poorly absorbed by the leaf (green) especially for thin leaves with a low pigment content (*Lactuca*). The reflexion and absorption coefficients are considerably reduced in this case.

(6) In infiltrated leaves the character of the curves of the reflexion and transmission coefficients with change in the angle of incidence of light is similar to the controls retaining the general shape and differing from it only quantitatively.

(7) The coefficient of absorption of light in infiltrated leaves, while remaining less than for the controls, rises with an increase in the angle of incidence.

(8) In leaves with autumn yellowing, there is a sharp change in the ratio of the optical parameters in the various regions of the spectrum as compared with normal leaves, but the curves of the reflexion, transmission and absorption coefficients and the angle of incidence of light are of the same character as in normal green leaves.

(9 In mean latitudes, the leaves of mesophytic plants are arranged at an angle of 45–50° to the horizon, adjusting themselves to the fullest absorption of the solar radiant energy incident on their surface at an angle from 0 to 60°.

(10) The vertical disposition of fluorescent tubes in light culture improves the light conditions for leaves of the lower strata but has no effect on the radiant energy utilization coefficient.

*Translated by* A. CROZY

VOL
5
196

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VOL. 5 1960

# DETERMINATION OF THE MEAN LIFE SPAN OF ERYTHROCYTES ENTERING AND CIRCULATING IN THE BLOOD*

YE. N. MOSIAGINA

Institute of Paediatrics, U.S.S.R. Academy of Medical Sciences, Moscow

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## CURVES OF CHANGE IN THE NUMBER OF TRANSFUSED, LABELLED ERYTHROCYTES

OF the utmost importance for the objective scientific study of haemolytic processes in the body is correct quantitative determination of the rate of destruction of erythrocytes in normal and pathological conditions. If the mean life span of the erythrocyte ($T$ days), he number of erythrocytes per cubic millimetre ($10^6$ $E$) and the total volume of circulating blood ($V$ litres) are known, then the daily number of erythrocytes destroyed equals 1000 $VE/T$.

The most widely used method for determining the life span of erythrocytes is transfusion of biologically or radioactively labelled erythrocytes [1, 2]. As is known, this method consists essentially in taking a sample of the erythrocytes from the circulating blood, labelling them by one means or another and re-injecting them either into the original donor or another recipient.

In recent years, a large number of highly interesting papers have been published on the mathematical interpretation of the curves of change in the number of transfused erythrocytes as a function of time [1–9]. Nevertheless, insufficient light has been cast on a number of factors associated with change and distribution of the life span of erythrocytes and there are still no simple methods for making an objective assessment of the curves of change in the number of transfused erythrocytes.

* Biofizika **5:** No. 3, 318–326, 1960.

Most work rs consider it advisable to investigate separately the effect of ageing and random destruction on the life span of erythrocytes. Accordingly, the concept of the potential life span of erythrocytes has been introduced, i.e. the span which would apply in the complete absence of random destruction and consequently the time before removal of erythrocytes from the circulation solely because of natural ageing. Although such a division may give an idea of the effect of individual factors on the life span of erythrocytes, it is somewhat artificial. In real conditions there is no hard and fast time of destruction due to ageing, since such destruction is also subject to certain chance factors: on the other hand, the probability of random damage to the erythrocyte is undoubtedly a function of its age and congenital makeup.

From the point of view of the quantitative study of destruction of erythrocytes, the determinant factor is not the potential lifetime of the erythrocyte, but the actual time of its existence. Therefore, hereafter, it is precisely this life span that we shall determine. It is most desirable to characterize the effect of random factors on the life span of the erythrocyte by introducing the generally-accepted indices of random distribution — the dispersion, the mean square deviation and the relative mean square deviation. In determining these values, it is necessary to know (together with the mean life span of the erythrocytes $T$) the mean square value $T_e$ of this life span on the basis of which the dispersion $\sigma^2$ is computed from the formula:

$$\sigma^2 = T_e^2 - T^2 \tag{1}$$

and the relative mean square deviation, i.e. the ratio of the mean square deviation $\sigma$ to the mean life span $T$ from the formula:

$$\frac{\sigma}{T} = \sqrt{\frac{T_e^2}{T^2} - 1}\,. \tag{2}$$

The value of this dispersion characterizes the effect of random factors on the deviation of the life span of the erythrocytes from the ideal potential life span. If we consider (nominally) separately the dispersion of the potentual life span (which we denote as $\sigma^2_{pot}$) and the dispersion ($\sigma_a^2$) due to intensified destruction of the erythrocyte through purely chance factors, then by the known theorem for the sum of dispersions we can write:

$$\sigma^2 = \sigma^2_{pot} + \sigma_a^2$$

$\sigma_a$ being determined from the formula:

$$\sigma_a = \sqrt{\sigma^2 - \sigma^2_{pot}} \tag{3}$$

The value $\sigma_{pot}$ may be taken as the mean on the basis of an investigation of a number of normal subjects. But bearing in mind that the ratio $\sigma_{pot}$ to $\sigma$ is small, it may be accepted as an approximation that $\sigma_a$ is equal to $\sigma$, i.e. the dispersion $\sigma^2$ is essentially an index of the destruction of the erythrocytes under the influence of random external factors.

Before embarking on a direct analysis of the fundamental meaning of the phenomena associated with the gradual disappearance of transfused, labelled erythrocytes and a determination of the mean and mean square life span of the erythrocytes ($T$ and $T_e$), we must deal with the following basic definition of the concept of the mean life span of erythrocytes, a concept which has not hitherto been defined in these terms in the literature.

In experiments with transfusion of labelled erythrocytes only those erythrocytes present at any given moment in the blood are taken from the donor. However, the mean life span of all the erythrocytes present at the given moment in the body (which we shall denote by $T_b$) is not equal to the actual mean life span of the erythrocytes ($T$), i.e. to the mean time elapsing from the appearance to the death of each erythrocyte. This difference arises from the fact that at any given moment, the presence in the blood of a particular erythrocyte is more likely the longer its life span.

In order to demonstrate this phenomenon more clearly, we shall consider a simplified theoretical scheme in which all the erythrocytes entering the blood may be divided in terms of life span into two numerically equal groups: we shall assume, for example, that 50 per cent of the erythrocytes live 50 days and 50 per cent 150 days. The mean life span of the erythrocytes will obviously equal $(50+150)/(2) = 100$ days. However, at any moment the erythrocytes of both groups are by no means present in the blood in equal numers, since the probability of the presence in the body of the erythrocytes with a life span of 150 days is 3 times as great as for those with a lifetime of 50 days. With a mean life span of 100 days, the relative number of erythrocytes with alife span of 150 days will be $0{\cdot}5\times(150)/(100) = 0{\cdot}75$ of their total number and with a life span of 50 days a total of $0{\cdot}5\times(10)/(100) = 0{\cdot}25$. Therefore, the mean life span of those erythrocytes which are present at any given moment in the blood is equal not to 100 days, but to $0{\cdot}75\times150+0{\cdot}25\times50 = 125$ days, i.e. 25 per cent greater than the actual mean life span.

In actual conditions, there are not two but a very large number of groups of erythrocytes with different lifetimes. If we assume that there are $n$ groups of erythrocytes with life spans $t_1$, $t_2$, $t_3$ ..... $t_n$ and the proportion of erythrocytes in each group in relation to their total number is respectively $\Delta q_1$, $\Delta q_2$, $\Delta q_3$ ..... $\Delta q_n$, then the actual mean life span of the erythrocytes ($T$) will obviously be equal to

$$T = t_1\,\Delta q_1+t_2\,\Delta q_2+t_3\,\Delta q_3+\ \ldots\ +t_n\,\Delta q_n. \tag{4}$$

The probability ($\Delta p$) of the presence in the blood at any given moment of time of erythrocytes with a life span $t_1$, $t_2$, $t_3$ .... $t_n$ will be equal respectively to

$$\Delta p_1 = \frac{t_1\,\Delta q_1}{T}, \qquad \Delta p_2 = \frac{t_2\,\Delta q_2}{T}, \qquad \Delta p_3 = \frac{t_3\,\Delta q_3}{T} \ldots \Delta p_n = \frac{t_n\,\Delta q_n}{T},$$

and the mean life span ($T_b$) of the erythrocytes present at a given moment in the body

$$\frac{T_b = t_1\Delta p_1+t_2\Delta p_2+t_3\Delta p_3+\ \ldots\ +t_n\Delta p_n = t_1^2\Delta q_1+t_2^2\Delta q_2+t_3^3\Delta q_3+\ \ldots\ +t_n^2\Delta q_n}{T}. \tag{5}$$

Since the numerator of the expression (5) for $T_b$ represents the square of the mean square value of the life span of the erythrocyte, we may write that $T_b = T_e^2/T = T(\sigma^2/T^2+1)$. Consequently, the greater the actual scatter in the life span of the individual erythrocytes, the greater will be the value $T_b$ with respect to $T$.

For practical purposes, it is of interest to establish both these values. As pointed out above, the mean life span $T$ determines the value of the daily destruction of erythrocytes, i.e. is the main criterion of the intensity of the haemolytic processes. The value $T_b$ is of importance for estimating the quality and duration of life of the blood transfused into the recipient.

Further consideration of the problem of defining the values $T$ and $T_b$ on the basis of the curves of change in the number of transfused erythrocytes is limited in this article to conditions in which the daily number of erythrocytes entering the blood and passing out of circulation remains unchanged during the entire period corresponding to the life span of the erythrocyte: in addition, we shall assume uniform conditions of life for the erythrocyte in both donor and recipient and we shall consider that blood transfusion itself has no appreciable influence on the life span of the erythrocyte.

## DETERMINATION OF THE LIFE SPAN OF ERYTHROCYTES PRESENT IN THE CIRCULATING BLOOD

To elucidate the phenomena occurring with varying life spans of the erythrocytes, we shall first consider the simplest and arbitrary case in which there are only a few groups of erythrocytes with different life spans. We shall assume, for example, that at the moment of transfer of blood there are $y_0$ erythrocytes consisting of three groups: the first comprising $y_1 = y_0 \Delta p_1$ erythrocytes with life span $t_1$ equal for all the erythrocytes of the given group; the second $y_2 = y_0 \Delta p_2$ with life span $t_2$; and, thirdly, the group $y_3 = y_0 \Delta p_3$ with a life span $t_3$. The values $\Delta p_1 = y_1/y_0$; $\Delta p_2 = y_2/y_0$; and $\Delta p_3 = y_3/y_0$ are the fractions of the numbers of erythrocytes of each group to the total number of erythrocytes $y_0$. It is clear that $y_1+y_2+y_3 = y_0$ and consequently $\Delta p_1+\Delta p_2+\Delta p_3 = 1$.

The gradual reduction in the number of erythrocytes in each group as a function of time is represented by straight lines since the life span within each group is uniform. Let the number of erythrocytes as a function of time be depicted for the first, second and third groups by the straight lines 1, 2 and 3 respectively with slopes $\alpha_1$, $\alpha_2$ and $\alpha_3$. It is clear from Fig. 1 that $\tan \alpha_1 = y_1/t_1$, $\tan \alpha_2 = y_2/t_2$ and $\tan \alpha_3 = y_3/t_3$.

The dependence on time of the change in the total number of erythrocytes may be obtained by summating the ordinates of lines 1, 2 and 3. We thereby obtain the broken line 4 composed of three straight line parts with different, gradually diminishing slopes. Thus, the tangent of angle $\beta_1$ is determined by the sum of the tangents of the angles of slope of all three lines 1, 2 and 3, etc.

$$\tan \beta_1 = \tan \alpha_1+\tan \alpha_2+\tan \alpha_3 = \frac{y_1}{t_1} + \frac{y_2}{t_2} + \frac{y_3}{t_3};$$

the tangent of angle $\beta_2$ (the slope to the second part of line 4) is defined as $\tan \beta_2 = \tan \alpha_2 + \tan \alpha_3 = y_2/t_2 + y_3/t_3$; and, finally, the tangent of the angle $\beta_3$ for the third part of line 4 equal to $\tan \beta_3 = \tan \alpha_3 = y_3/t_3$. The reduction in the slope of the various components of line 4 is due to a complete disappearance of the erythrocytes of the given group. Thus, change in the tangent of the angle of slope on passing from the first to the second part of line 4 is determined by the number of erythrocytes of the first group since $\tan \beta_2 - \tan \beta_1 = -\tan \alpha_1 = -y_1/t_1$.

The distribution of erythrocytes adopted by us into three groups with equal life spans of all erythrocytes in each group is, of course, purely arbitrary; in reality, the different erythrocytes have the most varied life spans and the number of groups with uniform duration will be very high. Therefore, line 4 should consist of a very large number of separate straight-line portions, i.e. in the limiting case should change into a smooth curve with gradually diminishing slope.

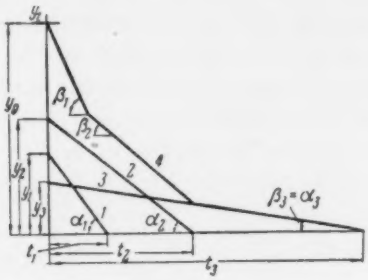


FIG 1.

Consequently, the number of transfused erythrocytes as a function of time must be represented by a curve with a downward directed convexity, this corresponds to the curves found experimentally. The gradual diminution in the slope of the curves is to be explained, as with the reduction in the slope of broken line 4 in Fig. 1, by the complete disappearance at any moment of time $t$ of the erythrocytes with a life span equal to $t$.

Thus, the experimental curves obtained by various workers relating the number of transfused erythrocytes in the circulating blood to time are of a quite regular character, readily explicable theoretically and fully attributable to two factors; the varying ages of the erythrocytes at the moment of withdrawal of blood and the different life spans of the erythrocytes.

Since, as a result of the enormous number of erythrocytes, the number of groups with different life spans is virtually infinite, we can formulate an equation for the change in the number of erthrocytes with time. In so doing, use must be made of the fact that change in the tangent of the angle of slope at any moment of time $t$ is equal to the proportion of erythrocytes with a life span equal to $t$ to this time.

Passing from finite values to infinitely small ones, we must replace the values $y_0 \Delta p$ by $y_0\, dp$ where $dp$ is the proportion of erythrocytes with life span $t$. Since the tangent

of the angle of slope to the axis of the abscissa of the curve relating the number of erythrocytes to time $y = f(t)$ is equal to the derivative $dy/dt$ of inverse sign, the general equation for the curve $y = f(t)$ may be derived from the condition that an infinitely small reduction in the tangent of angle of slope of this curve at moment $t$, i.e. the value $d(dy/dt)$, is equal to the ratio of the number of erythrocytes $y_0\, dp$ with lifetime $t$ to this time, i.e. equal to $y_0\, dp/t$.

Consequently,

$$d\left(\frac{dy}{dt}\right) = \left(\frac{y_0 dp}{t}\right),$$

whence

$$\frac{d^2y}{dt^2} = \frac{y_0}{t}\,\frac{dp}{dt} \tag{6}$$

The analytical expression for the curve $y = f(t)$ obtained allows us to define the mean life span and construct the curve of distribution of probability densities of the life span of the erythrocytes, since the values $dp/dt$ are the corresponding probability densities. For this purpose it is first necessary to construct from the experimentally plotted curve $y = f(t)$, the curve $dy/dt = f'(t)$ and then the curve $d^2y/dt^2 = f''(t)$ and finally, the probability density distribution of the life span of the erythrocytes, determined from expression (6) as:

$$\frac{dp}{dt} = \frac{t}{y_0}\,\frac{d^2y}{dt^2}. \tag{7}$$

From the curves $dp/dt = f(t)$, we can also determine the mean duration $T_b$ of life of the transfused erythrocytes, which is obviously equal o

$$T_b = \int_0^{T_{max}} t\,\frac{dp}{dt}\,dt, \tag{8}$$

where $T_{max}$ the longest life span of the erythrocytes, i.e. the time elapsing from the moment of transfusion of the erythrocytes to their complete disappearance from the circulating blood.

However, this mean life span $T_b$ can be much more easily solved directly from the curves $y = f(t)$ by solution of equation (8) substituting in it the value $dp/dt$ from equation (7).

The following expression is thus derived for $T_b$:

$$T_b = \int_0^{T_{max}} t \cdot \frac{t}{y_0}\,\frac{d^2y}{dt^2}\,dt$$

or

$$T_b = \frac{1}{y_0}\int_0^{T_{max}} t^2\, d\left(\frac{dy}{dt}\right) dt.$$

Integration by parts gives:

$$T_b = \frac{1}{y_0}\left[T_{max}^2 \left(\frac{dy}{dt}\right)_{t=T_{max}} - \int_0^{T_{max}} \frac{dy}{dt}\, 2t \cdot dt\right].$$

Since, because of the smoothness of the change in the life span of the erythrocytes, the derivative $(dy/dt)_{t=T_{max}}$ at the moment of time $t = T_{max}$ must be equal to zero, the first term of the expression in the square bracket is equal to zero and consequently:

$$T_b = -\frac{2}{y_0}\int_{y_0}^{0} t\, dy = \frac{2}{y_0}\int_0^{T_{max}} y\, dt. \qquad (9)$$

Thus, the overall mean life span of the transfused erythrocytes before and after transfusion is equal to double the area of the curve $t = f(y)$ or, what is the same thing, the curve $y = f(t)$ divided by the initial number of erythrocytes $y_0$.

The same conclusion can be derived directly from inspection of curve $y = f(t)$ on the basis of the following considerations. If an infinitely small number of erythrocytes $dy$ has a life span after transfusion of $t$ days, then the number of erythrocyte-days of these erythrocytes will be $tdy$ and the total number of erythrocyte-days $\int_0^{y_0} tdy$. But this number of erythrocytes is also equal to the initial number of erythrocytes $y_0$ multiplied by the mean duration $T'_b$ of their life after transfusion. Hence

$$T'_b y_0 = \int_0^{y_0} t\, dy, \quad \text{so that} \quad T'_b = \frac{1}{y_0}\int_0^{y_0} t\, dy$$

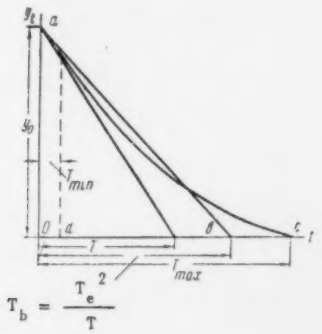


FIG 2.

Considering that, on average, the erythrocytes were taken from the blood halfway through their existence, as aking of blood occurs at a completely chance moment of life of the erythrocytes, we may assume $T'_b = 2T_b$, which leads to formula (9).

In order to obtain the mean duration $T_b$ of the life of the erythrocytes in the $y = f(t)$ diagram, it is necessary to draw from point $a$ (Fig. 2), corresponding to the initial number $y_0$ of transfused erythrocytes, the straight line $ab$ in such a manner that the area of the triangle $abo$ formed by line $ab$ is equal to the area of the curve $y = f(t)$.

Then, segment *ob*, equal to double the area of this triangle divided by $y_0$, will represent, according to formula (9), the mean life span of the transfused erythrocytes $T_b$. The greatest abscissa *oc* of the curve $y = f(t)$ depicts the longest life span of the erythrocytes $T_{\max}$.

The initial part of the function $y = f(t)$ may be rectilinear as shown in Fig. 2. This means that no erythrocyte has a total life span before and after transfusion, less than the time during which the function $y = f(t)$ is rectilinear. Consequently, segment *od* in Fig. 2, corresponding to the total duration of the straight line part of the function $y = f(t)$, represents the minimum duration of life of the erythrocytes $T_{\min}$.

As already stated, the difference in the life span of the erythrocytes largely depends on chance factors which play an important rôle in certain anaemic conditions. It is natural that random destruction affects the curve of probability density distribution of the life span, $dp/dt = f(t)$, giving a larger catter to this curve. However, direct comparison of the $dp/dt = f(t)$ curves is not sufficiently objective, and at the same time takes up a great deal of time and energy in constructing these curves. Therefore, as an objective criterion of the effect of chance factors on the character of the curve $y = f(t)$ it is desirable to use, as stated above, the dispersion $\sigma_b^2$ of the life span of the transfused erythrocytes. In accordance with formula (1) it is necessary in order to define $\sigma_b^2$ to find the mean square value of the life span of the transfused erythrocytes $T_{be}$ which in turn is determined from the formula:

$$T_{be}^2 = \int_0^{T_{\max}} t^2 \frac{dp}{dt}\, dt. \tag{10}$$

Substituting the value $dp/dt$ from formula (7), we get

$$T_{be}^2 = \frac{1}{y_0} \int_0^{T_{\max}} t^3 \frac{d^2y}{dt^2}\, dt = \frac{1}{y_0} \int_0^{T_{\max}} t^3 d\left(\frac{dy}{dt}\right).$$

Integration by parts gives:

$$T_{be}^2 = \frac{1}{y_0}\left[T_{\max}^3 \left(\frac{dy}{dt}\right)_{t=T_{\max}} - \int_0^{T_{\max}} \left(\frac{dy}{dt}\right) 3t^2\, dt\right],$$

or, taking into account that $dy/dt$ for $t = T_{\max}$ equals zero

$$T_{be}^2 = \frac{3}{y_0} \int_0^{y_0} t^2\, dt. \tag{11}$$

Consequently, to define the square of the mean square value of the life span of the erythrocytes circulating in the blood, it is necessary, by squaring the abscissa of curve $y = f(t)$, to construct the curve $t^2 = f(y)$ and to find the area of this curve. Trebling the area obtained and dividing it by the initial number of erythrocytes $y_0$, we get the value $T_{be}$ from which in accordance with formula (1) it is easy to compute the mean square deviation $\sigma_b$ and its ratio $\sigma_b/T_b$ to the mean life span of the erythrocytes.

## DETERMINATION OF EFFECTIVE LIFE SPAN OF ERYTHROCYTES

As shown earlier, the probability $\Delta_p$ of any life span $t$ of the erythrocytes present at a given moment in the circulating blood and the probability $\Delta_q$ of the same life span of the erythrocytes which have just entered the peripheral blood are connected by the relation

$$\Delta p = \frac{t}{T} \Delta q,$$

where $T$ is the actual mean life span of the erythrocytes.

Passing from finite to infinitely small values, we get

$$dp = \frac{t}{T} dq,$$

from which in accordance with formula (7):

$$\frac{dq}{dt} = \frac{T}{y_0} \frac{d^2y}{dt^2}. \tag{12}$$

From this formula we can on the basis of curve $y = f(t)$ construct the probability density distribution curve of the actual life span of the erythrocytes.

The actual mean life span of the erythrocytes $T$ may be found on the premise that the integral of the curve of probability density distribution $dq/dt = f(t)$ within the limits of 0 to $T_{max}$ must equal unity. Hence,

$$\int_0^{T_{max}} \frac{dq}{dt} dt = \int_0^{T_{max}} \frac{T}{y_0} \frac{d^2y}{dt^2} dt.$$

Since $d^2y/dt^2 = d/dt\,(dy/dt)$, then

$$\frac{T}{y_0} \left[ \left( \frac{dy}{dt} \right)_{t=T_{max}} - \left( \frac{dy}{dt} \right)_{t=0} \right] = 1.$$

Considering that $dy/dt$ for $t = T_{max}$ equals zero, we get

$$T = \frac{-y_0}{(dy/dt)_{t=0}}. \tag{13}$$

Thus, the actual mean life span of the erythrocytes is equal to the initial value of the number of transfused erythrocytes divided by the absolute value of the derivative of the curve $y = f(t)$ at its point of origin. Graphically, this value may be obtained as the segment cut off by the tangent on the axis of the abscissa at the point of origin of the curve $y = f(t)$, (Fig. 2).

* This formula coincides with that derived by another method by Dornhorst [4].

The square of the mean square value of the actual life span of the erythrocytes $T_e^2$ is defined by analogy with formula (10) by the following integral:

$$T_e^2 = \int_0^{T_{max}} t^2 \frac{dq}{dt} dt = \int_0^{T_{max}} t^2 \frac{T}{y_0} \frac{d^2y}{dt^2} dt .$$

Integrating by parts and substituting the limits, we get

$$T_e^2 = \frac{2T}{y_0} \int_0^{T_{max}} y d . \qquad (14)$$

Consequently, the value $T_e^2$ is equal o double the area of the curve $y = f(t)$ divided by the initial ordinate and multiplied by the actual mean life span of the erythrocytes $T$.

If we construct a triangle of area equal to that of curve $y = f(t)$, then the base of this triangle (*ob* in Fig. 2) will represent, in accordance with formula (14), the ratio $T_e^2/T$, which as follows from formula (5) is equal to $T_b$. The ratio of this value to $T$ is equal to $T_e^2/T^2 = (\sigma/T)^2+1$.

In the complete absence of elements of chance in the destruction of the erythrocytes, the life span of all erythrocytes would be equal, and the function $y = f(t)$ rectilinear. In this event $T_b = T = T_e$ and, consequently, $\sigma/T = 0$. With purely chance destruction of the erythrocytes, as is known, the function $y = f(t)$ is expressed by the exponential curve $y = y_0\, e^{-t/T}$ where $T$, as before, is the actual mean life span of the erythrocytes. It can be shown that in this case $T_b = 2T$, $T_e = \sqrt{2T}$ and $\sigma/T = 1$. Thus, depending on the intensity of random destruction of erythrocytes, the value $\sigma/T$ varies from 0 to 1.

It should be noted that in curves $y = f(t)$ the values of $y$ are most conveniently expressed as fractions of the initial value $y_0$, i.e. by constructing the relations $y/y_0 = f(t)$. Then, in all the formula computed above $y_0 = 1$ must be assumed, which leads to simplification of calculation in defining the life span of erythrocytes.

## SUMMARY

(1) Investigations into the process of gradual destruction of transfused, biologically or radioactively labelled erythrocytes have shown that the life span of all the erythrocytes entering the peripheral blood from the bone marrow, and the mean life span of the erythrocytes present at any given moment in the circulating blood, have different values.

(2) To characterize the degree of scatter of the life span of the erythrocytes, it is proposed that instead of the indices at present in use (fractions of the random daily destruction or the period of reduction by half of the number or radioactivity of the transfused erythrocytes) the criterion of the relative mean square deviation commonly adopted for analysis of random factors be applied.

(3) A method is given for determining the above-mentioned values characterizing the life span of the erythrocytes from the curves of decline in the number of transfused, labelled erythrocytes with time.

(4) A method is given for constructing the curves of distribution of probabilities of the life of the erythrocytes on the basis of the curves of the decline in the number of transfused erythrocytes with time.

*Translated by* A. CROZY

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VOL. 5 1960

# PROBLEM OF BIOMECHANICAL ASYMMETRY AND ITS CORRECTION ON APPLICATION OF PROSTHETICS*

I. SH. MOREINIS

Central Research Institute for Prosthesis Application and Construction, Moscow

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IT is known that in the living body there is constant maintenance of certain physico-mechanical values which are automatically regulated. The stable position of the human body in the field of gravity is ensured by the anti-gravitational automatisms controlling the skeletal musculature and enabling man "not to think" about his balance.

Man "does no think" either when walking since here too the effect of automatisms converting walking into a dynamic stereotype is manifest.

According to Bernshtein's figurative descrip ion; "Walking is a very complex co-ordination of structure involving the entire body musculature... and drawing it into one complete process of movement... As in an orches ra each instrument plays its individual score, so in the act of walking each joint reproduces its own curve of movements and each centre of gravity performs its sequence of accelerations... And in like manner the whole of this ensemble works in unison with a single and complete rhythm, fusing the whole enormous complexity into clear and harmonic simplicity" [1].

With due caution, one may attempt to equate the activity of the living body and the work of technical automatic devices.

In justification of posing the problem in this way we would allude to the statement of V. I. Pavlov: "Man is, of course, a system (roughly speaking — a machine) like

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any other in Nature and subject to inevitable laws which are the same for all Nature, but a system, which at the level of our present scientific vision, is unique in its highest self-regulation. We are already quite familiar with a variety of self regulating machines produced by human hands. From this point of view the method of study of the system — man — is the same as any other system: resolution into parts, study of the significance of each part, study of the relation of parts, study of the links with the environment and, finally, understanding, if this is within the means of man, its general work and regulation on the basis of all this...."

We shall consider from the standpoint of "resolution into parts" the human act of walking as he co-ordination of simpler movements — elements of movements. The elements of movement of the lower limbs on walking may be classified, for example, in the following manner:

(1) Movement of limb in transfer phase. The limb is regarded as a double physical pendulum, with the point of support at the centre of the hip joint set in motion according to a definite law.

(2) Rotation of the body around the axis parallel to the supporting plane and perpend cular to the direction of walking in the limb support phase.

(3) Rotation of the body around the vertical axis passing through the centre of support of the limb in the support phase, and turning of the limbs in the support and transfer phases around their long axes (turning of limbs with respect to pelvis).

(4) Rotation of the body around an axis corresponding to the direction of movement and passing through the centre of the hip joint of the supporting limb.

It is of interest to submit to verification and analysis the general postulates on the regulation of symmetry on natural experimental material — after amputation — a problem of great practical importance for the theory of prosthesis. From the point of view of the complex approach to the application of prosthesis after amputation of the lower limbs, three problems may be raised: (a) fulfilment of the function of support and movement; (b) fulfilment of the function of contraction of the lost or excized muscles; and (c) fulfilment of the functions of cutaneous and kinaestheric sensations developed in the removed part.

Problem (c) may be solved by cybernetics. The most general plan for solution of this problem, given that (a) and (b) have already been solved, is that outlined in the paper by Weiner [2].

Problem (a) is approximately solved in practice by the experimental findings of physiologists and prosthetic practitioners. The problem has received less study from the standpoint of mechanics. If we ignore the reasons for the appearance of interaction forces between the component parts of the human body, then walking may be looked upon as the totality of movements in space of the links of the supporting-motor apparatus of man. This assumption allows us to apply equations of motion for a solid body in order to define a number of analytical relations characterizing walking.

Before amputation, the qualitative and quantitative characteristics of the elements of movement on taking a step with the left or right limbs are uniform. A certain asymmetry in the elements of movements on taking a step with the left or right limbs

occurs also in normal conditions. We assume it to be small as compared with the asymmetry appearing after amputation when the values of the displacements, velocities and accelerations for similar elements of movement become different for the healthy and amputated sides. Thus, walking becomes asymmetrical. We suppose that a direct connexion exists between the main problems of prosthetic application and the

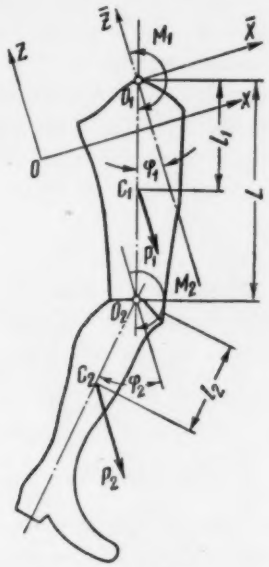


FIG 1. Model of lower limb in transfer phase.

principle of symmetry; problems (a) and (b) are associated with the maximum possible degree of restoration of the geometric, static and dynamic symmetry, problem (c) with restora ion of kinaesthetic symmetry. Hence, all the problems of prosthetic application may be reduced to a single one—restoration of symmetry. The following assumption can also be made; minimum asymmetry must be realized for maximum retention of the habitual automatisms ensuring stereotyped walking with one artificial limb. To illustrate this point, we shall first consider the first element of movement (Fig. 1). To simplify, we shall consider the straightforward problem of a double physical pendulum where the thigh is the first and the shin and foot the second member.

The equations of motion, in the form of Lagrange, have the following form;

$$\begin{aligned}
&\alpha_{11}\ddot{\varphi}_1+\alpha_{12}\left[\ddot{\varphi}_2\cos(\varphi_1-\varphi_2)+\dot{\varphi}_2^2\sin(\varphi_1-\varphi_2)\right]+\\
&+c_{11}\left[\frac{1}{g}(\ddot{z}\sin\varphi_1-\ddot{x}\cos\varphi_1)+\sin\varphi_1\right]=M_1;\\
&\alpha_{21}\left[\ddot{\varphi}_1\cos(\varphi_1-\varphi_2)-\dot{\varphi}_2^2\sin(\varphi_1-\varphi_2)\right]+\varphi_{22}\ddot{\varphi}_2+\\
&+c_{22}\left[\frac{1}{g}(\ddot{z}\sin\varphi_2-\ddot{x}\cos\varphi_2)+\sin\varphi_2\right]=M_2;
\end{aligned}\qquad(1)$$

where

$$\alpha_{11} = I_{0,y} + \frac{1}{g} P_2 L^2; \quad \alpha_{12} = \alpha_{21} = \frac{1}{g} P_2 L l_2;$$

$$\alpha_{22} = I_{0,y}; \quad c_{11} = P_1 l_1 + P_2 L; \quad c_{22} = P_2 l_2; \tag{2}$$

$P$, the weight of the human; $P_1$ and $P_2$, the weight of the first and second members; $M_1$ and $M_2$, the moments of force about the points of pivot; $l_1$ and $l_2$, the distance from the pivoting points to the centres of gravity; $i_1$ and $i_2$, the radii of the inertia of the members; $L$, the distance between the pivoting points; $L_1$, the length of the second member; $I_{0,y}$ and $I_{0,y}$, the moments of inertia of the members; $\varphi_1$ and $\varphi_2$, the general co-ordinates — angles of deviation of the axes of the members from the vertical; $x = x(t)$ and $z = z(t)$, the equations of motion of the point of pivot 0 — the centre of the hip joint.

For the further investigation of equations (1), we shall define the character of certain biomechanical values. From the findings of Bernshtein [3], Zal'tsgeber [4] and also the Central Research Institute for Prosthesis Applica ion and Construction and the Institute of Anthropology of the Moscow State University [5], it is known that

$$P_{1h} = (0{\cdot}125 \pm 0{\cdot}014)\, P; \; P_{2h} = (0{\cdot}061 \pm 0{\cdot}010)\, P;$$
$$l_{1h} = (0{\cdot}387 \pm 0{\cdot}032)\, L; \; l_{2h} = (0{\cdot}490 \pm 0{\cdot}022)\, L_1;$$
$$L_1 = (1{\cdot}100 \pm 0{\cdot}022)\, L. \tag{3}$$

In (3) and hereafter the subscript "$h$" indicates that the values under consideration apply to the healthy limb; index "$a$" to the artificial limb.

We were able to establish that

$$i_{1h} = (0\,417 \pm 0{\cdot}004)\, L \text{ and } i_{2h} = (0{\cdot}622 \pm 0{\cdot}010)\, L_1. \tag{4}$$

From the viewpoint of the probability theory and mathematical statistics, these mean values may be termed biomechanical constants, if we indicate the fiduciary intervals for each of them. We shall refer to the work of Bernshtein [3] from which it follows that the mean square deviations for the linear values $l_1$ and $l_2$ and consequently for $i_1$ and $i_2$ do not exceed $\pm 0{\cdot}03$. For the masses of the members these deviations are of the same order.

Using (3) and (4) it is easy to see that

$$\frac{\alpha_{11}}{c_{22}} = 2{\cdot}255\,\frac{L}{g}; \quad \frac{\alpha_{12}}{c_{22}} = \frac{L}{g}; \quad \frac{\alpha_{22}}{c_{22}} = 0{\cdot}805\,\frac{L}{g}; \quad \frac{c_{11}}{c_{22}} = 2{\cdot}936\,. \tag{5}$$

The constants in (5) are combinations of the biomechanical constants of (3) and (4).

On the basis of equations (5), equations (1) may be written in the following form:

$$\frac{L}{g}\left[2{\cdot}255\,\ddot{\varphi}_1 + \varphi_2 \cos(\varphi_1 - \varphi_2) + \dot{\varphi}_2^2 \sin(\varphi_1 - \varphi_2)\right] +$$
$$+ 2{\cdot}936\left[\frac{1}{g}(\ddot{z}\sin\varphi_1 - \ddot{x}\cos\varphi_1) + \sin\varphi_1\right] = \frac{M_1}{P_2 l_2};$$

$$\frac{L}{g}[\ddot{\varphi}_1 \cos(\varphi_1 - \varphi_2) - \dot{\varphi}_2^2 \sin(\varphi_1 - \varphi_2) + 0{\cdot}805\,\varphi_2] +$$

$$+ \frac{1}{g}(\ddot{z} \sin \varphi_2 - \ddot{x} \cos \varphi_2) + \sin \varphi_2 = \frac{M_2}{P_2 l_2}. \tag{6}$$

We shall consider two models, one of which imitates the movement of the healthy, and the other the artificial, limb in the transfer phase. We require that in the "$a$" model, relations (5) be observed and also

$$L_a = L_h = L$$

$$\frac{M_{1a}}{P_{2a} l_{2a}} = \frac{M_{1h}}{P_{2h} l_{2h}} \quad \text{and} \quad \frac{M_{2a}}{P_{2a} l_{2a}} = \frac{M_{2h}}{P_{2h} l_{2h}} \tag{7}$$

If we assume that the laws of motion of the points of suspension $O_{1h}$ and $O_h^1$ (centres of the hip joints) be uniform, i.e. $x_h = x_a$ and $z_h = z_a$, then the equations of motion (6) for the two models are identical. Consequently, for equal initial conditions,

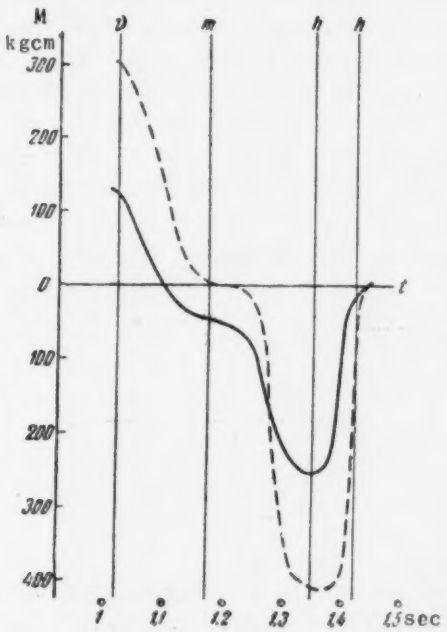


FIG. 2. Graph of moment about centre of hip joint interval of change, time of transfer; continuous line, calculated; dotted line, experimental.

the motions of the artificial and healthy limbs, at least formally, become symmetrical. Conditions (5) and (7) may therefore be interpreted as follows; in the artificial limb (stump + prosthesis) desirable ratios expressed by biomechanical constants must be observed. The requirement that conditions (5) and (7) be observed determines the

design for the construction of the prosthesis with reference to the individual biomechanical, anthropometric and anatomical features of the person in need of the appliance. In fact in conditions (7) the equality

$$L_a = L_h = L$$

is obvious. The equality

$$\frac{M_{2a}}{P_{2a}l_{2a}} = \frac{M_{2h}}{P_{2h}l_{2h}}$$

becomes the criterion for defining the parameters of the shin-hinged mechanism which must confer moment $M_{2a}$ on the prosthesis with the parameters of the shin $P_{2a}$ and $l_{2a}$ for the individual requiring the artificial limb, in whom the parameters for the healthy shin $P_{2h}$ and $l_{2h}$ apply. The value $M_{2h}$ may be found experimentally [6]. We would note, in passing, that the equations (6) can be utilized as formulae for computing the values $M_1$ and $M_2$ if we take as known $\varphi_1$, $\varphi_2$, $x$, $z$, $P$ and $L$.

For the calculation of $M_1$, for example, we make use of the $\varphi_1$, $\varphi_2$, $x$ and $z$ curves plotted as a result of statistical and probability treatment of a sufficiently large number of experimental findings [7]. We define the required first and second derivatives by means of the interpolation formulae of Newton [8]. Fig. 2 gives the $M_1$ curve for $L = 40$ cm, $L_1 = 51$ cm, $P = 70$ kg for an interval covering the time of transfer. For comparison, a dotted line is used to depict the curve for the muscular moments in the hip joint in the transfer phase of the walking step in normal conditions, taken from Bernshtein [3], being the result of a particular experiment.

The common character of the curves is quite obvious. Conditions (5) in the developed form lead to a set of equations defining the distribution of mass in the hip prothetic

$$P_{2a} = \frac{1}{A_1}\left(\frac{P_{sh}l_{sh}}{L} - A_2\frac{P_{sh}i_{sh}^2}{L_2}\right) + \frac{1}{A_1}\left(\frac{P_{ch}l_{ch}}{L} - A_2\frac{P_{ch}i_{ch}^2}{L^2}\right)$$

$$l_{2a} = \frac{1}{A_3}\left[\frac{1}{P_{2a}}\left(\frac{P_{sh}l_{sh}}{L}\right) + \left(\frac{P_{ch}l_{ch}}{L}\right) + 1\right]$$

$$\left(\frac{i_{2a}}{L_1}\right)^2 = A_4\frac{l_{2a}}{L_1} \qquad (8)$$

where $P_{2a}$, the weight of the artificial shin together with foot and footwear; $l_{2a}$, the distance of the centre of gravity of the artificial limb from the axis of the knee joint; $i_{2a}$, the radius of inertia of the ar ificial shin; $P_{sh}$, $l_{sh}$, $i_{sh}$, the analogous characteristics for the hip stump; $P_{ch}$, $l_{ch}$, $i_{ch}$, the characteristics for the artificial casing of the hip; the constants $A_i$, the combinations of biomechanica constants.

For the practical utilization of equations (8), it is necessary in each case to determine the characteristics of the hip stump.

The complexity of this task can be overcome if use is made of an approximated formulae from which the vertical shift in the centre of gravity of the human body

occurring as a result of amputation ($\Delta z$) is obtained. The relation between the level of amputation and the magnitude of the vertical shift of the general centre of gravity of the individual was obtained experimentally and is expressed in the curves in Fig. 3 taken from the work of Krivanek and Petak [9]. Derivation of these formulae is based on the assumption that there exists a linear relation between the shortening of the

FIG. 3. Displacement of general centre of gravity in relation to level of amputation. Abscissa, healthy leg; ordinates, upward displacement. Levels of amputation; (1) exarticulation of hip; (2) short; (4) medium; (6) long; (7) exarticulation of knee.

hip as a result of amputation, the position of the centre of gravity and the size of the radius of inertia of the hip stump, on the one hand, and the vertical shift of the general centre of gravity, on the other. On the basis of the above remarks the formulae for computing the weight of the stump and also for defining the position of the centre of gravity and the radius of inertia may be represented in the following form (in the formulae atrophy of the stump is taken into account [10]):

$$\frac{P_{sh}}{P_a} = \frac{195 \times 10^{-5}(95 - \Delta z)}{1 + 195 \times 10^{-5}(95 - \Delta z)};$$

$$\frac{l_{sh}}{L} = \frac{0{\cdot}387}{41}(91 - \Delta z) \qquad (9)$$

$$\frac{i_{sh}}{L} = \frac{0{\cdot}417}{41}(95 - \Delta z),$$

where $P_a$, the weight of the individual with amputated leg.

We shall compare the calculated values for $P'_{sh}$ and $l'_{sh}$ obtained from formulae (9) w th the experimental values $P''_{sh}$ and $l''_{sh}$.

Taking into account the experimental errors of weighing and measurement, the calculated figures may be taken as quite satisfactory.

Formulae (9) render amenable to calucation the parameters determining the distribution of mass in the prosthesis according to formulae (8). Since for the six parameters

$P_{2a}$, $l_{2a}$, $i_{2a}$, $P_{ch}$, $l_{ch}$, $i_{ch}$ there are three equations, only three of them can be independent and the system (8) gives broadly speaking a countless multiplicity of solutions. To obtain the real solutions, it is necessary to resort to additional considerations deter-

TABLE 1

| Subject | $L$ (mm) | $P_e$ (kg) | $z$ (mm) | $P'_{sh}$ (kg) | $P''_{sh}$ (kg) | $l'_{sh}$ (mm) | $l''_{sh}$ (mm) |
|---|---|---|---|---|---|---|---|
| N | 400 | 64·2 | 63 | 3·81 | 4·03 | 115·30 | 100 |
| Sh | 450 | 54 | 74 | 2·13 | 2·30 | 88·95 | 90 |
| Z | 430 | 58 | 66 | 3·08 | 2·80 | 114·60 | 105 |

mining the choice of independent parameters. Such a detailed analysis of equations (8) is not the intention of this article and we shall only refer to one of the variants as shown in Fig. 4 by way of illustration.

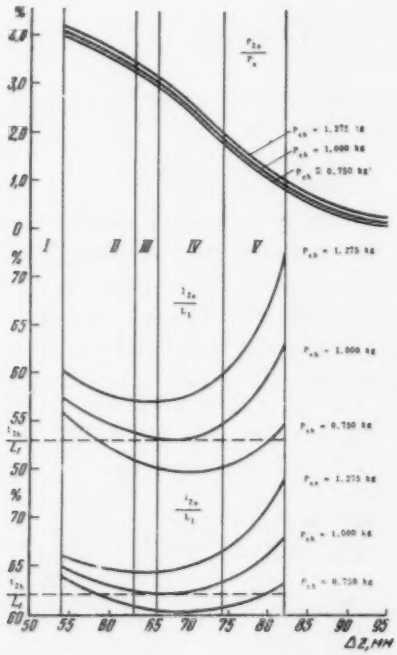


FIG. 4. Calculated characteristic of inertia of second member for given parameters of first member. Upper curves, relative weight; central curves, position of centre of gravity; lower curves, radius of intertia. I, II, III, IV, V, levels of amputation from — exarticulation of knee to short.

In order to compare the calculated data with the experimental results, we shall use the results of work carried out in the Central Research Institute for Prosthesis Application and Construction under the direction of A. N. Kabanov. The application

of physiological and clinical tests demonstrated experimentally that change in the weight of the hip casing, has little effect on the function of walking with an artificial limb: the parameters of the distribution of mass must be considered separately for the hip casing and for the shin with foor; the distance of the centre of gravity of the shin with foot and footwear from the knee joint must not exceed 60–65 per cent of the length of the second member; the distance of the centre of gravity in the hip casing from the knee joint must be not less than 40 per cent of the length of the first member; the radius of inertia of the shin must not exceed the distance to the centre of gravity by more than 150 mm. It is easy to see that these experimental recommendations while not being of a differentiated character, do not diverge from the average calculated data.

VOL. 5 1960

## SUMMARY

(1) As a result of amputation in man, geometric, statico-dynamic and kinaesthetic symmetry is disturbed. One of the problems of application of a prosthesis is to lessen this asymmetry.

(2) The mathematical expression for the principle of symmetry in prosthetics application is the observance of the required ratios expressed by biomechanical constants. Observance of these ratios leads to an identity of the equations of motion for the healthy and artificial sides. Some of these constants were known and some ($i_{1h}$ and $i_{2h}$) have been redefined.

(3) The ratios mentioned above must include the inertia characteristics of the stump, determination of which in each particular case by experimental means creates difficulties. The complexity of this problem is overcome by approximated analytical relations for determining the weight, position of centre of gravity and the radius of inertia for the hip stump. These analytical relations give quite satisfactory agreement with the results of direct weighing and measurements.

(4) A variant for solving the problem of mass distribution in the hip prosthesis is considered. Analysis of the solution shows that the regular tendency for the weight of the members of the prosthesis to fall is not self purposive and it must be u ed for the simplest way of achieving a rational mass distribution.

(5) The results of the analytical solution of the problem of mass distribution in the hip prosthesis are in good agreement with the findings of experimental investigations. The analytical recommendations are of a differentiated character and reflect the individual peculiarities of the person requiring an artificial limb.

*Translated by* A. CROZY

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VOL
5
196

# EXISTENCE OF SEVERAL TYPES OF LATENT DAMAGE IN IRRADIATED MYOSIN MOLECULES*

L. Kh. Eidus and Ye. E. Ganassi

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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The damage inflicted by radiation on biological objects is not always directly manifest during irradiation. In many cases the phenomenon of "after-effect" is observed, i.e. additional destruction occurring after cessation of irradiation. Thus, a number of workers (for review see [1]) have detected injury to irradiated objects continuing for a long time after i radiation (hours and even days), under the action of comparatively low temperatures which are not in the least harmful to unirradiated objects. The distinctive manifestations of this after-effect, in particular, the similar course of the process in biological and model protein systems, indicate that it is of a physicochemical nature, in which the effect of heat is manifest otherwise than by affecting the rate of the m tabolic processes. The quantitative relation between the scope of damage during irradiation and with the "after-effect" which we shall nominally call the "heat" after-effect, varies in different cases and depends on the test object and conditions of the experiment.

Recently, a new phenomenon also leading to intensified radiation injury as a result of post-radiation effects has been discovered [2–4]. It has been demonstrated in model experiments [2] that oxygen acting after irradiation on anaerobically irradiated myosin partially inactivates it and that the proportion of activity lost depends on the radiation

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dose. It has also been shown that on irradiation in the absence of oxygen inactivation generally does not occur until doses are reached which in aerobic conditions would produce 50 per cent inactivation of the myosin. However, on irradiation, a certain "excited" state is formed which may respond to interaction with oxygen. This state persists *in vacuo* for hours and even days. Subsequent admission of oxygen to the anaerobically irradiated protein leads to a sharp inactivat on which is only slightly less, taken as a whole, than that produced by irradiation in air. Immediately after addition of oxygen there is an almost instantaneous loss of activity of a definite value determined by the radiation dose. A similar "oxygen" after-effect has also been noted in certain living objects [3, 4] and in this case, as in the case of the "heat" after-effect, the characteristics of the process (similar manifestation in model and living systems, instantaneous effect of oxygen, etc.) point to the physicochemical nature of the processes in which the oxygen effect occurs — otherwise than via participation in the metabolic processes.

VOL. 5 1960

In our model experiments [2], it was particularly evident that the damaging effect of oxygen is due to the physical mechanism of its interaction with the protein molecule somehow activated by irradiation.

The physical nature of these "excited" states of the macromolecules responsible for the phenomenon of after-effect in our experiments and, apparently, in experiments on living objects, has not yet been conclusively established and will be the subject of further research. It is difficult to decide whether we are dealing here with "frozen" free radicals, with "trapped" unpaired electrons, or with a different form of disturbance in the electron structure of the protein macromolecule.

The aim of the present work was to bring us nearer an understanding of these phenomena and, in particular, to ascertain whether in the phenomena of radiation after-effect, the "point of application" of heat and oxygen is identical or differs, and whether these agents action the same or different foci of latent damage. Experiments have been undertaken with alternation of the effect of heat and oxygen on solutions of myosin previously irradia ed wi h diff.rent doses of $^{0}$Co $\gamma$-rays in anaerobic conditions (after removing dissolved air with a vacuum pump).

The criterion of radiosensitivity, was the extent to which the ATPase activity was affected. The anaerobically irradiated myosin solution was divided *in vacuo* into two parts, and one was heat-inactivated anaerobically (the activity of the myosin was determined by the method described in [2]). Subsequent admission of oxygen caused additional inactivation. To the second part of the irradiated protein, oxygen was admitted which produced partial inactivation ("oxygen" after-effect and only then was the protein heat-treated. This is schematically depicted in Fig. 1. Incubation was carried out at temperatures of 20–27° which do not affect the intact protein but inactivate that part which, as a result of irradiation, passes into a state with a low heat-inactivation barrier. It was found that after anaerobic incubation of irradiated solutions, admission of oxygen immediately caused additional inactivation (bc in Fig. 1). The measurements made in 15 parallel experiments with different radiation doses showed that the value of the "oxygen step" was not dependent on preliminary

heat treatment. The ratio of the values of the "step" before and after incubation (in the Fig.) this in the ratio of the lengths of the segments a–d—b–c) was equal to $1{\cdot}1 \pm 0{\cdot}1$. On the other hand, admission of oxygen after anaerobic irradiation while inactivating part of the myosin did not remove the causes of the "heat" after-effect which occurred after this in the same way as in anaerobic conditions. These experiments demonstrate the independence of the "heat" and "oxygen" after-effects. The points of application of heat and oxygen differ. In other words, in the myosin molecule irradiated but retaining enzymatic activity, there are at least two types of centres of latent damage responsible for the sensitivity of such a molecule to the additional external factors — heat and oxygen.

The formation of these centres occurs during irradiation. The question arose as to whether oxygen is involved in their formation. In our experiments, the myosin was irradiated in a salt water solution and therefore the oxygen effect could make itself felt in various ways including via the $HO_2$ radical formed on radiolysis of the wate .

The rôle of oxygen in the formation, during irradiation, of the centres of latent damage subject to heat treatment (more precisely, responsible for the "heat" after-effect) could be established by comparing the proportion of enzymatic activity lost as a result of the "heat" after-effect following aerobic and anaerobic irradiation.

The myosin solution was accordingly divided into two parts. One was freed from dissolved air, after which the entire protein was irradiated with $\gamma$-rays at a dose corresponding to 50 per cent inactivation in aerobic conditions. After irradiation both portions of solution, containing and not containing air, were subjected to heat-inactivation and then the activity of the myosin in them was determined. The duration of their incubation was chosen with reference to the temperature to ensure that the heat after-effect was fully manifest and no further loss of act vity would occur. A total of 12 experiments was carried out. It was found that the fall in activity as a result of the heat after-effect on irradiation in air ($\Delta A_1$ in the Fig.) was the same as on irradiation in anaerobic conditions ($\Delta A_2$ in the Fig.). The ratio $\Delta A_1/\Delta A_2 = 1{\cdot}0 \pm 0{\cdot}1$. Hence it follows that just as many "heat" centres are formed in aerobic conditions as in anaerobic, i.e. oxygen does not take part in thei formation either via the $HO_2$ radical or another route.

It follows from the above results that oxygen does not take part in the formation of the greater part of the centres responsible for the "oxygen" effect. In fact, as the Fig. shows, the greater part of the complete "oxygen effect" (a—e) is accounted for by the "oxygen" after-effect (a—d) and the damage due to the presence of oxygen during irradiation itself (d—e) does not exceed two-fifths of the whole effect.

The existence, discovered in our experiments, of two different types of centres of latent damage in the protein myosin molecule is apparently closely connected with the phenomena observed by the method of electron paramagnetic resonance (e.p.r) on irradiation of various protein substance . As has recently been shown [5] the e.p.r spectra appearing on irradiation of dried preparations of proteins indicate the existence in the protein molecule structure of two types of unpaired electron "traps", the rela-

tion between which varies in different protains. One type of trap may be attacked by the molecular oxygen of the medium and the corresponding e.p.r. spectrum is thereupon modified or may even vanish. At the same time the unpaired electrons in the trap of the other type, do not interact with oxygen and the spectrum they give persists for a long time not only *in vacuo* (as in the first case) but in aerobic conditions.

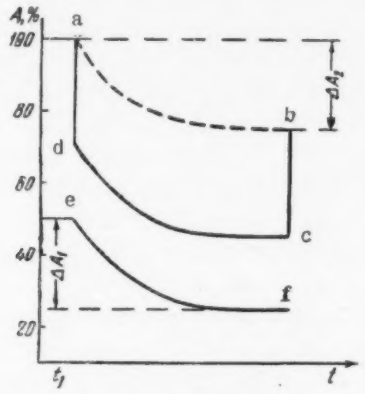


FIG. 1. Character of "oxygen" and "heat" after-effects on irradiation of myosin solution. Ordinates, enzymatic activity (A); abscissa, time after irradiation (t); ($t_1$) start of incubation; (ef) heat inactivation of myosin irradiated aerobically by a dose corresponding to 50 per cent inactivation "under the beam". Fot the same dose anaerobic irradiation does not give a loss in activity (point "a") (abc) anaerobic heat inactivation of this solution (ab), with subsequent admission of oxygen (bc); (adc) admission of oxygen after anaerobic irradiation (ad), with subsequent heat inactivation (dc).

We discovered the existence of various types of latent damage in one and the same protein molecule by demonstrating the independence of the post-radiation effect of two agents — heat and oxygen. The joint effect of these factors in the range of positive temperatures has previously been studied on other objects by Stapleton and Edington [6] and Neary [7] and it would be natural to compare their results with ours. But the first work was concerned with elucidating the rôle of temperature and oxygen tension during irradiation itself, and the existence of the after-effect reaction and its relation to these factors was not taken into account. However, there are grounds for believing that the dependence of radiosensitivity on temperature found in these experiments was due not to he direct radiation effect, but to the heat after-effect. In this case the results would be comparable with ours.

In fact, it is known that in a number of cases radiation damage is intensified with increase of temperature during irradiation. The dependence of the di ect radiation effect on temperature is unexpected and hard to explain, but it can be understood if it is assumed that it is caused essentially by the "heat" after-effect. Direct damage at the moment of irradiation apparently does not depend on temperature, but on irradiation, some of the molecules of the substrate pass in o a long-lived state with a reduced heat-inactivation barrier. If the temperature during irradiation is sufficiently

high, then these molecules are responsible for further damage to the object, summating with the direct damage, and this is the cause of the temperature dependence of the entire radiation reaction.

In the experiments of Stapleton and Edington oxygen increased the damage to *E. coli* ("oxygen effect") at all temperatures at which irradiation was performed, whereas the radiosensitivity varied very sharply with temperature. If in accordance with the above-mentioned observations we assume that the change in sensitivity was due to the after-effect reaction, then these results fully accord with the independence of the effect of heat and oxygen found by us at the molecular level.

Also in the experiments of Neary, the specific effects of heat and oxygen were studied. The author heated to 20°, seedlings of *Vicia fabia* first irradiated in the cold (1–2°) in anaerobic and aerobic conditions and compared the effects of heating in the presence and absence of oxygen. The final result, as in our experiments, did not depend on the sequence of heat and oxygen treatment. (It should be noted, however, that the results of these experiments do not substantiate ours since the scheme of the experiment adopted by the author ruled out a separate study of the rôle of heat and oxygen in the after-effect).

Thus, the few experiments carried out in this direction on other objects are not in conflict with our conclusions on the independent effect of heat and oxygen on the latent radiation damage.

The results of our series of experiments aimed at defining the rôle of oxygen in the production of latent damage, can also be compared with the corresponding findings of other workers. Thus, in the experiments of Anderson [8] the heat after-effect was observed on irradiation of solutions of pepsin in the absence of $O_2$ (in a nitrogen atmosphere), this is in agreement with the idea that oxygen is not involved in the production of the "heat" centres of latent damage. However, according to the author, the after-effect on irradiation of pepsin in an atmosphere of oxygen was even more considerable than in nitrogen. However, in our view, this still does not prove that oxygen participates in the formation of certain portions of the "heat" centres, since the same effect could be induced by an "oxygen" effect greater than in the case of nitrogen.

We would also note that in solutions of DNA the after-effect has been observed only on irradiation in the presence of oxygen [9].

## SUMMARY

(1) It has been shown that on anaerobic irradiation, there are formed, at least, two different types of latent damage in myosin molecules.

(2) Heat or oxygen acting on the anaerobically irradiated myosin cause additional inactivation ("heat" or "oxygen" after-effect). Each of these factors acts only on one of the two types of latent damage and does not act on the other, therefore, with simultaneous application of heat and oxygen the post-radiation effect is summated.

(3) Oxygen present in the medium during irradiation of myosin does not take part (either via the $HO_2$ radical or by other routes) in the formation of latent damage

subject to the "heat" after-effect. Nor does it take part in formation of that latent damage (at least the majority of it) responsible for the "oxygen" after-effect.

(4) Attention is drawn to the close analogy of the results obtained and those of investigations of irradiated proteins by the method of electron paramagnetic resonance.

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# FORMATION OF STABLE ORGANIC PEROXIDES IN THE LIVING BODY AS A RESULT OF IONIZING RADIATION*

I. K. Kolomiitseva

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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Study of the formation of lipid peroxides resulting from the irradiation of animal tissues is of interest in attempting to elucidate the mechanism of action of radiation on biological objects. It is known that lipid peroxides introduced into the body have high toxicity and mutagenic properties [1, 2]. It is also known that after irradiation of lipids *in vitro* in the presence of $O_2$ there is a chain-reaction type rise in the amount of peroxides [3–6]. These facts have served as the basis for postulating the production on irradiation of weakly reactive lipid peroxides which exert a toxic effect as they accumulate in the lipids of the body [1, 7, 8].

However, these concepts have still not been supported by sufficient experimental evidence. Firstly, increase in the content of lipid peroxides in the living body immediately

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after irradiation with near-lethal doses cannot be considered a firmly established fact. The main experimental findings of a rise in the content of peroxides in the lipids of the body after irradiation are given in paper [7], the au hors of which investigated, by colorimetric methods, the peroxide content in *n*-butanol extracts of intact carcasses of mice after irradiation to a dose of 950 r.

In the first experiments, concerned with investigation of the peroxide content in irradiated mice at different times after irradiation (up to 7 days), the authors observed no increase. Judging from the results reported, the variations in the experiment lie within the limits of the variations of the controls. After the method had been somewhat modified (instead of MgSO as drying agent 0·18 N LiCl was used) in the experiment with leucoindophenol in aerobic conditions on ten animals (five irradiated and five controls) a distinct increase was obtained in the amount of peroxides in the extracts of the tissues of the irradiated mice; absence of peroxides (0·00 μM peroxide per g mouse body weight) in the controls, and 0·22 μM after irradiation.

In a subsequent series of experiments the authors used leucoindophenol catalysed by copper as the reagent in aerobic conditions. In these experimental conditions, a difference in the content of peroxides between the irradiated and unirradiated mice was found on heating the samples to 100°; at room temperature there was a uniform amount o peroxides. In the extracts of unirradiated and irradiated mice coloration was found to be depressed at certain concentrations of LiBr and LiCl. The authors observed a similar phenomenon on oxidation of Cu — indophenol by a solution of para-haematin. However, spectroscopic investigation of the extract for chromogens did not give positive results. Despite this, the authors do not exclude the possibility of detection in this case of a certain active compound of the para-haematin type.

It should be noted that in the absence of LiCl, the peroxide content in the extracts of irradiated and unirradiated mice showed virtually no difference even on heating.

As evidence of the peroxide nature of the compounds determined, the authors refer to experiments in which determination of the peroxides was made with $SnCl_2$ in anaerobic conditions, with extraction by 0·5 M LiCl in *n*-butanol. These experiments gave a statistically reliable (difference between experiment and control 3 $\sigma$) increase in the content of peroxides in the extracts from he mouse carcasses immediately after irradiation with a dose of 950 r. In the extracts of the unirradiated mice, 0·42 μM peroxide was determined per g mouse body weight. As stated above, in the extraction with leucoindophenol, no peroxide was found in the extracts of the unirradiated mice although all the peroxides known in these conditions give a distinct reaction with leucoindophenol. The conditions of performing the reaction with $SnCl_2$ were shifted to the alkaline side by use of 0·1 M $LiCOOCH_3$ and this undoubtedly increased the possibility of the reaction of $SnCl_2$, on heating for 5 min at 100°, with weak oxidizing agents of a non-peroxide nature (iron, haematin derivatives, etc).

The conflicting results obtained by these authors make it impossible to say with certainty that there is any increase in peroxides in the animal body immediately after irradiation.

Of the other studies of the problem of the origin of lipid peroxides in the animal tissues under the influence of radiation, reference must be made to an investigation [9] showing an increase in the amount of peroxides in the skin immediately after radiation with a dose of 20,000 r. However, as Philpot [1] stresses, it is not clear whether this increase came about in the living or dead skin. The peroxides were found only for doses of 20,000 r, i.e. when their formation in the free fat was possible. At doses of 1000 r the formation of peroxides immediately after irradiation was not detected. There are also references to an increase in the peroxides in the fats of individual rat organs at different times after irradiation.

For example, an increase in peroxides in the fats of the rat testes examined 30 min after irradiation with a dose of 800 r has been reported [10]. The authors do not report the results of the experiments, merely pointing out that in the controls, negative values were obtained; increase in the experiment was 0·38 μM peroxide per g fat. However, Chevallier himself in a subsequent paper [11], states that the negative values in the controls make necessary a very cautious approach to the results obtained.

VOL. 5 1960

In the report of Tarusov [12], reference is made to the experiments of Zhuravlev (unpublished) demonstrating an increase in the content of peroxides in the liver fats on the 4th–5th day after irradiation of rats with a dose of 700 r. However, the absence of factual experimental information and the references to repetition of the experiment also rule out an assessment of the degree of reliability of the increase.

An increase in peroxides in the fatty tissue of rats has been found in vitamin E deficiency [13]. An attempt to use vitamin E as protective substance on irradiation did not give positive results [14].

Thus, at present, no convincing evidence has been produced in support of the notion of an increase in the peroxide content in the animal body immediately after irradiation. This situation lent interest to a more careful investigation of the content of lipid peroxides in the normal and irradiated animal organism.

## EXPERIMENTAL METHODS

For an experimental solution of the problem we used the highly sensitive method proposed by Hartmann and Glavind [15] of oxidation of leucoforms of 2:6-dichlorophenol indophenol by peroxides.

Checking of this method on the peroxide of succinic acid, synthetically prepared by us, demonstrated 100 per cent detection of the peroxide by this method. The least amount of peroxide of succinic acid giving distinct coloration (on measurement with an FEK electrophotocolorimeter was 0·01 μM per ml of sample.

However, aerobic treatment of the test tissue and subsequent aerobic determination has a number of serious shortcomings. The danger of peroxide formation during treatment of the test material is not fully excluded and the initiation of chain reactions of oxidation on heating with leuco-dye is possible. This may lead to an erroneous conclusion on the true peroxide content of the tissues. Therefore, in parallel with the aerobic method of oxidation we used an anaerobic method in which oxidation of the leuco-dye and colorimetry occurred in an atmosphere of nitrogen in neutral

medium (pH 7) in the presence of lithium acetate. To exclude the appearance of artefacts during treatment, the tissues were frozen with liquid nitrogen, ground up and extracted in purified *n*-butanol. Parallel determination of peroxides by the aerobic and anaerobic methods enabled us to distinguish the so-called non-reactive peroxides (of the type of hydroperoxides of unsaturated fatty acids) from the reactive ones (succinic acid peroxide type). Thanks to the anaerobic modification applied by us (oxidation of the leuco-stain in neutral medium) it proved highly sensitive and was instrumental in detecting react ve peroxides in a concentration of $10^{-8}$ M per ml sample.

Since we obtained calibration curves on pure peroxide of succinic acid, the amount of peroxides was later expressed as its equivalents. All the operations were performed in *n*-butanol purified from pre-formed peroxides by distillation from a solution containing $SnCl_2$ and $H_2SO_4$. The methods used were as follows.

*A. Aerobic method of determination*

1·5 ml test solution was mixed with 1 ml 0·1 M lithium acetate solution in butanol, 0·1 ml of 0·001 N leuco dye solution in butanol and 0·1 ml glacial acetic acid, and the mixture heated at 100° for 10 min. The solution was rapidly cooled to 0°, its extinction determined in a cuvette on the FEK with a green filter at 524 mμ.

*B. Anaerobic method of determination*

1 ml test solution and 2·5 ml 0·5 N lithium acetate solution in pure *n*-butanol was placed in the main part of a Thunberg vessel. Into the side arm was poured 0·5 ml 0·001 M solution of leuco 2:6-dechlorophenol indophenol. Nitrogen with oxygen removed was passed through the vessel for 10 min [16], then the vessel was closed, the fluids mixed and heated for 5 min a boiling water bath. After cooling in the flow of nitrogen the fluid was transferred to the FEK cuvette and measured with a red filter.

Before continuing to an investigation of the animal tissues, the peroxides in certain auto-oxidized lipids were determined. The results obtained are set out in Table 1.

It is clear from the results that the aerobic method always gives a raised content of peroxides as compared with the anaerobic one, the ratio of these values being close to 2·8 in the case of reactive peroxides (butanol peroxide) and as much as 5 for non-reactive peroxides (cholesterol peroxide).

TABLE 1. DETERMINATION OF ORGANIC PEROXIDES IN AUTO-OXIDIZED LIPIDS

| Substance | Aerobic method (μM/g) | Anaerobic method (μM/g) | Ratio Aerobic/ anaerobic |
|---|---|---|---|
| *n*-butanol after irradiation with 500,000 r | 14·6±1·2 | 5·3±0·32 | 2·8 |
| Purified oleic acid | 23 | 6 | 3·8 |
| Oleic acid after storage in air | 215±20 | 65±5 | 3·3 |
| Purified linoleic acid | 37±3 | 8·6±0·3 | 4·3 |
| Cholesterol | 32±2 | 6·5±0·5 | 4·9 |

It was also of interest to investigate the sensitivity of the aerobic and anaerobic methods on determining the peroxides produced by irradiation in any organic substance for example, in *n* butanol. Experiments were thus carried out to determine the peroxides in *n*-butanol after irradiation at doses from 760 to 9600 r. The *n*-bu anol was irradiated in glass vessels closed by cellophane, with a RUP unit (180 kV, 10 mA without filte , dose rate 387 r/min).

TABLE 2. RELATION BETWEEN DOSE OF RADIATION AND AMOUNT OF AEROBICALLY AND ANAEROBICALLY DETERMINABLE PEROXIDES IN *n*-BUTANOL

| Dose (r) | Aerobic method | Anaerobic method |
|---|---|---|
| | μM perioxide per g *n*-butanol | μM peroxide per g *n*-butanol |
| 0 | 0·07±0·007 | 0·02 |
| 760 | 0·085±0·008 | 0·023 |
| 3200 | 0·13 ±0·005 | 0·025 |
| 6400 | 0·20 ±0·02 | — |
| 9600 | 0·27 ±0·01 | 0·09 |
| 500,000 | 14·6 ±1·2 | 5·3±0·32 |

Judging from the curve of formation of peroxides in *n*-butanol in relation to dose, the aerobic indophenol method revealed an increase in the content of peroxides in *n*-butanol after irradiation at doses above 1000 r. The anaerobic indophenol method gave an increase in the peroxides in the irradiated sample only with a dose of 10,000 r (Table 2).

The results show that the methods applied are sufficiently sensitive to determine changes in the peroxide content in the lipids of the irradiated body.

In investigations of the peroxide content of the carcasses of irradiated and unirradiated rats, males were used weighing 110–150 g. Irradiation to a dose of 1000 r was carried out with a RUP X-ray unit (180 kV, 10 mA, dose rate 42 r/min, Cu 0·5 mm+ Al 0·75 mm). Immediately after irradiation, the animal was killed by decapitation and homogenized for 5 min with 100 ml 0·5 M LiCl in *n*-butanol. The mixture was filtered and 1·5 ml of filtrate used to determine the peroxides in aerobic conditions, as described above. We give the results;

DETERMINATION OF PEROXIDES IN WHOLE RAT CARCASSES IMMEDIATELY AFTER IRRADIATION TO A DOSE OF 1000 r (μM peroxide per g tissue)

Unirradiated* 0·08, 0·01, 0·06, 0·02, 0·05, 0·04, 0·06, 0·02, 0·02, 0·01
Irradiated * 0·06, 0·06, 0·03, 0·01, 0·02, 0·01

* Mean value of peroxide content 0·03±0·02.

The peroxide content of the intact rat carcasses was very low (not more than 0·08 μM per g tissue) and gave a large scatter of values. Immediately after irradiation with a dose of 1000 r, no appreciable change in the peroxide content in the carcasses occurred.

Homogenization of the intact animal is very difficult owing to the large size of rats as compared with mice. From our observations, the skin is insufficiently ground

up in an homogenizer. It could also be assumed that contact with the metallic knives of the homogenizer helps to destroy the peroxides. Therefore, the next series of experiments was performed on young rats weighing 25–30 g. After decapitation of the animal the skin was removed, the carcass and skin fixed by liquid nitrogen, weighed and ground up seperately in a porcelain mortar and then extracted with 0·5 M LiCl for 10 min with agitation in a shuttle apparatus. The determination was made in the same way as before. We give the results;

DETERMINATION OF PEROXIDES IN CARCASSES OF YOUNG RATS (WITHOUT SKIN) IMMEDIATELY AFTER IRRADIATION AT A DOSE OF 1000 r (μM peroxide per g tissue)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Unirradiated* | 0·13 | 0·10 | 0·14 | 0·04 | 0·04 | 0·03 | 0·03 | 0·06 |
| Irradiated† | 0·05 | 0·07 | 0·06 | 0·04 | 0·08 | 0·08 | 0·03 | 0·07 |

* Mean value of peroxide content $0{\cdot}07 \pm 0{\cdot}04$
† ,, ,, ,, ,, ,, $0{\cdot}06 \pm 0{\cdot}01$.

DETERMINATION OF PEROXIDES IN SKIN OF YOUNG RATS IMMEDIATELY AFTER IRRADIATION AT A DOSE OF 1000 r (μM peroxide per g tissue)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Unirradiated* | 0·10 | 0·10 | 0·14 | 0·16 | 0·25 | 0·08 | 0·34 | 0·25 |
| Irradiated† | 0·19 | 0·18 | 0·13 | 0·11 | 0·09 | 0·22 | 0·28 | 0·22 |

* Mean values of peroxide content $0{\cdot}15 \pm 0{\cdot}07$
† ,, ,, ,, ,, ,, $0{\cdot}17 \pm 0{\cdot}05$.

As can be seen, the peroxide content in the carcasses of the gaung rats scarcely diverged from that in the tissues of the adult rats ($0{\cdot}03 \pm 0{\cdot}02$ μM/g in the adult rats and $0{\cdot}07 \pm 0{\cdot}04$ μM/g in the goung rats). In the skin of the goung rats, the peroxide content was somewhat higher than in the carcass. However, in neither the carcasses nor the skin of the infant rats did peroxide content increase immediately after irradiation with 1000 r.

Since it was still impossible to infer from the absence of increase in the peroxide in the intact carcass that changes in the peroxide content in the various organs are absent after radiation, it was of interest to investigate the peroxide content in certain rat organs immediately after irradiation. For this purpose, 5 g of the test organ was fixed with liquid nitrogen, ground up, weighed and extracted with an equal volume of 0·5 M LiCl in *n*-butanol. The subsequent treatment was as described for the young rats. The results are given in Table 3.

The peroxide content of the extracts of liver, testes, intestines, brain and muscles was very low—at the threshold of the sensitivity of the method. The peroxide content of the blood extracts was greater. It could be assumed that in the presence of LiCl the haem is split off from the haemoglobin, and partial oxidation occurs in the haematin due to the atmospheric oxygen. Experiments with crystalline haemoglobin of human blood obtained by us, showed that on extraction of haemoglobin with 0·5 M LiCl in *n*-butanol, the extract did not contain substances capable of oxidizing the leucoindophenol in either aerobic or anaerobic conditions.

TABLE 3. DETERMINATION OF PEROXIDES IF RAT ORGANS IMMEDIATELY AFTER IRRADIATION BY 1000 r

| Organ | Unirradiated | | Irradiated | |
|---|---|---|---|---|
| | No. of tests | μM peroxide per g tissue | No. of tests | μM peroxide per g tissue |
| Liver | 3 | 0·02 | 3 | 0·00 |
| Testes | 2 | 0·00 | 2 | 0·00 |
| Intestines | 3 | 0·02 | 4 | 0·00 |
| Brain | 4 | 0·01 | 3 | 0·01 |
| Muscles | 5 | 0·02 | 6 | 0·01 |
| Skin (aerobic determination) | 10 | 0·94±0·24 | 2 | 1·00 |
| Skin (anaerobic) | 3 | 0·40 | — | — |
| Blood (aerobic determination) | 17 | 0·12±0·07 | 12 | 0·12±0·06 |
| Blood ( naerobic) | 3 | 0·03 | — | — |

In the skin extracts of the adult rats, the ratio of aerobically determined peroxides and those in anaerobic conditions was 2·3. This indicates the reactive nature of the skin peroxides.

As Table 3 shows, in none of the organs investigated was the amount of peroxide immediately after irradiation with a dose of 1000 r increased. Repeating (in the methodological sense) Horgan and Philpot's work [7] we were unable to obtain in rats the increase in the amount of peroxides after irradiation observed by these authors in mice. As already emphasized, analysis of the data presented by these authors shows that they contain contradictory and unclear points excluding a well-defined conclusion.

Thus, the results of our work do not confirm the supposition that immediately after irradiation to near lethal doses, a measurable amount of weakly reactive organic peroxides is produced in the animal body.

## SUMMARY

(1) The aerobic method of Hartmann and Glavind as modified by Philpot and Horgan and the anaerobic method in our modification, were appl ed to determine the peroxides in auto-oxidized lipids. It has been shown that the value of the ratio of the aerobically determined peroxides and the anaerobic values differs for different peroxides and characterizes the reactivity of the peroxide. The lowest value of the ratio taken as 1 was obtained for succinic acid peroxide; the greatest for the peroxides of auto-oxidized cholesterol — 4·9.

(2) Determination of the peroxides in irradiated *n*-butanol showed that with the aerobic indophenol method an increase in the amount of peroxides is detected after irradiation with a dose above 1000 r.

(3) The anaerobic indophenol method was used to determine the peroxides in intact carcasses of rats weighing 110–150 g and in the carcass without the skin of young rats weighing 25–30 g. It has been shown that the peroxide content in the carcasses immediately after irradiation with a dose of 1000 r does not increase.

(4) It has been demonstrated by this method that in the liver, testes, intestines, brain and muscles the peroxide content is very low and is at the threshold of the sensitivity of the method (0·00–0·12 μM peroxide per g tissue).

(5) The blood peroxides of rats were determined both in aerobic (0·12±0 07 μM per ml blood) and anaerobic (0·03 μM peroxide per ml blood) conditions.

The value of the ratio of the aerobically determined peroxides to the anaerobic ones for blood was 4. This indicates the low reactivity of the blood peroxides.

(6) The peroxide content in the skin of adult rats was much greater (0·94±0·24 μM peroxide per g skin) than in the skin of yourg rats (0·15±0·07 μM peroxide per g skin). The value of the ratio of the aerobically determined peroxides to the anaerobic value for the skin of adult rats was 2·3, which is suggestive of the more reactive nature of the skin peroxides.

(7) The amount of peroxides determined in the rat organs immediately after irradiation with 1000 r did not exceed the peroxide content in the organs of the unirradiated animals.

The author wishes to express her appreciation to Prof. A. M. Kuzin for guidance and help in carrying out the work and for a discussion of the results.

*Translated by* A. CROZY

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# EFFECT OF IONIZING RADIATION ON PROTEIN METABOLISM IN NUCLEI, MITOCHONDRIA AND ERGASTOPLASM OF THE PLANT ORGANISM*

N. B. STRAZHEVSKAIA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL. 5 1960

IONIZING radiation has the effect of disturbing the functional activity and morphological structure of the cell organella. A number of studies on the animal organism point to the very high radiosensitivity of the process of oxidative phosphorylation in mitochondria [1, 2]. Recently, Il'ina [3] found deep disturbances in the protein metabolism in the nuclei of the liver and mucosa of the small intestine of rats in the period of acute radiation sickness; this was expressed in a sharp change in the relative quantitative content of all the proteins of the cell nucleus. Toropova [4] showed that the fall in the total weight of the isolated nuclei after irradiation, is paralleled by a decline in the DNP and DNA in the nuclei of the rat liver and that the nuclei after irradiation are enla·ged with a granular structure.

Butler, Cohn and Crathorn [5] found a slight increase in the incorporation of $^{14}C$ labelled phenylalanine in the proteins of the nuclei, mitochondria, microsomes and the supernatant fluid of the rat liver 1 hr after irradiation at a dose of 500 r. Similar findings were obtained by Richmond, Ord and Stocken (6) on the uptake of $^{14}C$ labelled glycine and lysine by the proteins of the mitochondria and microsomes of the thymus, liver and pancreas of rats 2 hr after irradiation (1000 r). The experiments conducted on plant organisms by Breslavets and Afanas'eva [7] showed that the diameter of the nuclei and number of mi oses in the cells of rye grains increased after irradiation at a dose of 750 r but with an increase in the radiation dose the number of mitoses fell, although the diameter of the nuclei remained larger than in the unirradiated cell. Sisakian [8] observed depression of the protein synthesis in rye seedlings 24 hr after irradiation at a dose of 10,000 r and more, which is in agreement with our findings on incorporation of $^{14}C$ labelled glycine and $D_2O$ into the proteins of wheat seedlings [9].

It follows from these published findings that it is s i l impossible to draw any definite conclusions on the place occupied by disturbances in protein metabolism in radiation injury to the cell as a whole, not to mention cell structures. Yet, for an understanding of the mechanism of action of ionizing radiation such investigations are unquestionably of great importance.

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The purpose of the present work was to study the effect of $^{60}Co$ γ-rays at a dose of 10,000 r on protein content and metabolism both in the cell as a whole and in the cell fractions of the plant organism (nuclear, mitochondrial and ergastoplasm fractions).

## METHODS

The object of the investigation made it necessary to choose and, if need be, modify the technique of isolating the cell organella. Isolation of the nuclei from plant tissue presents much greater difficulties than from animal tissues. This probably accounts for the small number of papers devoted to isolation and study of the chemistry and biochemistry of cell nuclei in plants [10–14]. It must be noted that in four of these papers the authors isolated the nuclei from dried embryos of rye, wheat and other cereals [10, 11, 13, 14].

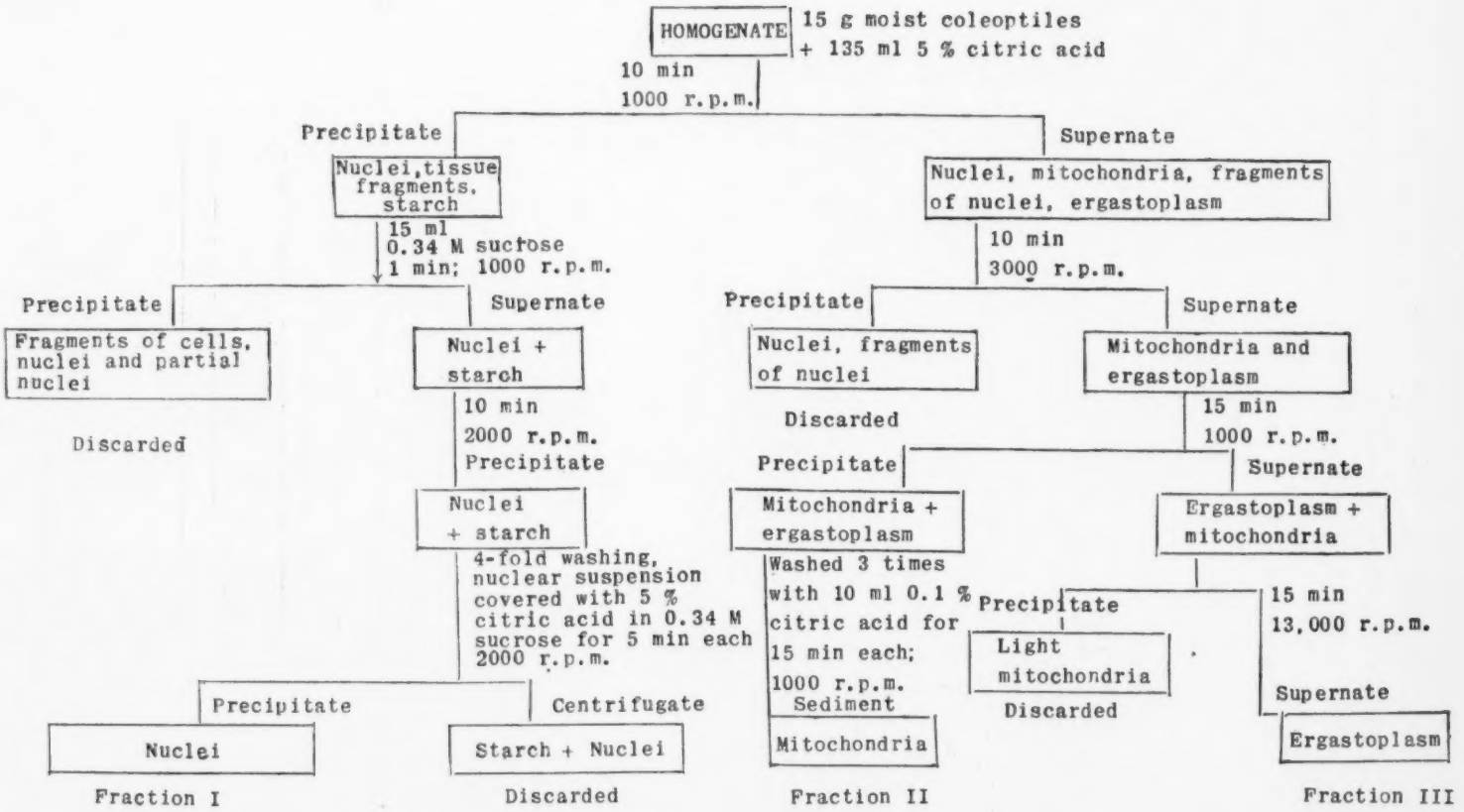
We used young wheat coleoptiles in which the physiological state of the nucleus is undoubtedly different from that in the resting embryo. This apparently is an explanation of the fact that none of the aqueous methods in the papers quoted [12–14] was suitable for our purposes. We tested the following media for isolating the undamaged nuclei: (1) 0·25 M sucrose +0·001 M $CaCl_2$; (2) 0·88 M sucrose +0·001 M $CaCl_2$; (3) 80 parts of 3% $K_2Cr_2O_7$ + 20 parts commercial formalin; 4) phosphate buffer pH 6·47 + formalin (4:1) + 0·01 M $CaCl_2$; (5) 2 M sucrose + 0·01 M $CaCl_2$; (6 1% citric acid; and (7) 5% citric acid.

In the first four media the nuclei were completely destroyed. In the fifth medium they were not destroyed but were few in number. The best quality and number of nuclei were obtained by the last procedure, i.e. in 5% citric acid and therefore, we adopted this solution.

The test object was 3-day old Leutescens 062 variety wheat seedlings grown in the dark in an incubator at 26° in distilled water. The seedlings were irradiated at a dose of 10,000 r $^{60}Co$ γ-rays (dose rate 500 r/min, uniformity of dose field ±5%) and after a day were placed for 24 hr in $^{35}S$ labelled methionine solution (100 ml, spec. activity 2·13 μc/ml; pH 6·8). Then (48 hr after irradiation) the coleoptiles were cut and weighed. Coleoptiles weighing 15 g (experiment or controls) were first finely cut with scissors in 135 ml 5% citric acid and then homogenized for 5 min. The homogenate was filtered through six layers of gauze and three fractions isolated from it: nuclear (I), mitochondrial (II) and ergastopla m (III) (see diagram). The purity of the fractions was checked microscopically by staining the preparations with acetocarmine and methyl green pyronin. The nuclear fraction was slightly contaminated with starch (10–15%) which it was impossible to get rid of completely even after repeated washing and centrifugation. The whole operation of isolating the cell fractions was performed in the cold at 3–5°. From each fraction, the proteins were precipitated with an equal volume of 10% trichloroacetic acid (TCA) washed three times with 1% TCA, twice with 96% alcohol and twice with ether. We had earlier demonstrated that irradiation results in an increase of the sorbing power of protein [9] in relation to $^{14}C$ labelled glycine, hence we re-precipitated our protein fractions in a surplus of unlabelled me hionine (1% methionine solution in 0·2% NaOH) for 18 hr. Repeated re-precipitation

VOL. 5 1960

SCHEME OF FRACTIONATION



was not necessary since after the second precipitation there was no change in activity. After re-dissolving, the proteins were precipitated with an equal volume of 10% TCA, washed, as in the preceding case, dried and weighed. Then the nucleic acids were extracted from the proteins, by Schneider's method [15], with 5% TCA for 15 min at 90°. We made a 3-fold extraction since once was insufficient (after the second extrac ion the activity increased). With such treatment in hot TCA, the starch also passed into solution. Then, the proteins were cooled, centrifuged, washed twice with 5% TCA, twice with alcohol and twice with ether and again weighed.

The activity, in counts per min, was determined on air-dried stems in the I–III fractions after re-precipitation in excess methionine and extraction with hot TCA.

The total proteins of the stems were isolated in the following manner: 5 g moist stems were ground with quar z sand in a small amount of 0·2% NaOH in a mortar and then extracted by shaking with ten times the volume of 0·2% NaOH for 2 hr. The suspension was centrifuged. The opalescent supernate was decanted and precipitated with an equal volume of 10% TCA. Then the protein precipitate was centrifuged, washed three times with 1% TCA, twice with alcohol and twice with ether. The dried proteins, as described above, were re-precipitated in a surplus of unlabelled methionine and then extracted three times with 5% TCA at 90° for 15 min each, washed and dried in a vacuum desiccator, 3–4 samples, each of 1 mg, were weighed on an analytical balance, spread on small brass plates (diameter, 1·8 cm) and counted with an end-window counter.*† For the uniform distribution of the sample over the plate, 8–10 drops of alcohol were added and then evaporated.

## RESULTS

The index of the rate of protein synthesis in our experiments was the incorporation into the proteins of $^{35}S$ labelled methionine. In a former paper we showed [16] that irradiation causes disturbance in root permeability. Therefore, in each experiment the activity was determined from the methionine $^{35}S$ in the irradiated and unirradiated coleoptiles which were used for isolating the cell fractions. Table 1 shows that the activity of the dried coleoptiles of the unirradiated and irradiated seedlings was similar.

Simultaneously with the isolation of the proteins from the cell fractions, the total proteins were isolated from the unirradiated and irradiated coleoptiles. The results

TABLE 1. UPTAKE OF $^{35}S$ METHIONINE BY IRRADIATED AND UNIRRADIATED WHEAT SEEDLING COLEOPTILES

| Samples | Activity, counts/min/mg dried coleoptiles (1, 2, 3, number of experiment) | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Controls | 1935±120 | 1164±2 | 1624±80 |
| Tests | 1884±160 | 1120±5 | 1626±80 |

* The samples were always measured in strictly standard geometric condition.

† In 24 hr the seedlings absorbed from the solution about 90 per cent of the original 35 S labelled methionine.

of these experiments are given in Table 2 and they show that synthesis of the total protein (as shown by the incorporation of $^{35}S$ methionine) did not change 48 hr after the action of γ-rays at a dose of 10,000 r.

TABLE 2. ACTIVITY OF TOTAL PROTEINS OF UNIRRADIATED AND IRRADIATED COLEOPTILES

| Samples | Activity, counts/min/mg protein (1, 2, 3, number of experiment) | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Controls | 3330±200 | 2959±3 | 982±60 |
| Tests | 3378±165 | 2986±20 | 987±120 |

VOL. 5 1960

In Table 3 are given the results of experiments on the uptake of $^{35}S$ methionine by the proteins of the fractions of the irradiated and unirradiated coleoptiles before and after extraction of nucleic acids. It should be noted that spectrophotometric determination at a wave length of 260 mμ of the DNA content in the hot TCA extracts from the nuclear fraction showed that in the irradiated coleoptiles the DNA was half that in the unirradiated stems. In the last column of Table 3 are given the mean values (of four experiments) of the percentage change in incorporation of $^{35}S$ methionine in the tests as compared with the controls. Comparison of the results before extraction of the nucleic acids w th those after, shows convincingly that before extraction, the proteins contained ballast substances, in particular, starch; consequently, the findings on protein activity after extraction of nucleic acids are more significant. It is interesting that protein synthesis in all the fractions was virtually unchanged. These find ngs are in agreement with those of the above-mentioned papers of Butler *et al.* [5] and Richmond *et al.* [6] who also found a certain increase in the uptake of labelled amino-acids by the proteins of the cell fractions of rat organs.

It is clear from Table 3 that the least relative activtiy after extraction of nucleic acids was given by the proteins of the ergastoplasm fraction both in tests and controls. Taking this activity as unity, we can calculate the relative activity of the proteins of the other fractions (Table 4).

Table 4 shows that in the unirradiated coleoptiles the relative activities of the proteins of the nuclear and mitochondrial fractions were very similar, although in the nuclear fraction the relative protein activity was slightly higher than in the mitochondrial.

In the irradiated coleoptiles we see a different picture: the highest relative protein activity was found for the mitochondrial fraction. It follows that the effect of γ-radiation is to reduce the rate of incorporation of $^{35}S$ methionine into the nuclear proteins and raise the rate of incorporation of $^{35}S$ methionine into the proteins of the mitochondrial fraction.

Our re ults (Tables 3 and 4) only permit estimation of the rate of incorporation of $^{35}S$ methionine into the proteins of the various cell fractions in normal conditions and after irradiation, but do not indicate the proportion of protein in these fractions to that in the whole cell. Our calculations listed below show that the ratio of the protein

TABLE 3. ACTIVITY (ACCORDING TO $^{35}S$ METHIONINE) OF PROTEINS OF ISOLATED FRACTIONS OF UNIRRADIATED AND IRRADIATED COLEOPTILES (COUNTS/MIN/MG PROTEIN). (+) SIGN DENOTES PERCENTAGE INCREASE: (—) SIGN, PERCENTAGE DECREASE

| Fraction | Experiment 1 | | | Experiment 2 | | | Experiment 3 | | | Experiment 4 | | | Mean percentage difference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Controls | Test | % difference | Controls | Test | % difference | Controls | Test | % difference | Controls | Test | % difference | |
| **Before extraction of nucleic acids** | | | | | | | | | | | | | |
| Nuclei | 1517±20 | 1214±2 | —20 | 877±10 | 658±18 | —27 | 1043±60 | 982±4 | —7 | 1071±30 | 1195±50 | +11 | (—11)±5·2 |
| Mitochondria | 2698±60 | 3142±60 | +16 | 1354±30 | 1622±40 | +20 | 1640±15 | 2128±100 | +30 | 1598±5 | 2427±10 | +51 | (+29)±9·5 |
| Ergastoplasm | 2129±80 | 2457±10 | +12 | 1311±80 | 1078±60 | —18 | 1859±60 | 1859±140 | 0 | 2473±1 | 2345±40 | —5 | (—3)±5 |
| **After extraction of nucleic acids** | | | | | | | | | | | | | |
| Nuclei | 4113±20 | 2971±50 | —28 | 1942±70 | 1932±60 | —3 | 3058±30 | 2066±10 | —33 | 2905±100 | 3450±40 | +19 | (—11)±4 |
| Mitochondria | 3407±80 | 3704±80 | +9 | 1969±1 | 2352±50 | +20 | 1640±70 | 2140±140 | +35 | 3828±250 | 3472±50 | +14 | (+19)±6·2 |
| Ergastoplasm | 1845±100 | 2168±50 | +17 | 1755±80 | 1469±80 | —16 | 1385±35 | 1773±100 | +28 | 2751±200 | 3808±260 | +38 | (+16)±7·1 |

VOL. 5 1962

amounts in the various fractions in the cell in normal conditions and after irradiation sharply changes.

TABLE 4. RELATIVE ACTIVITIES OF PROTEINS OF ISOLATED FRACTIONS OF UNIRRADIATED AND IRRADIATED COLEOPTILES (AVERAGE DATA OF FOUR EXPERIMENTS)

| Samples | Fraction | | |
|---|---|---|---|
| | Nuclei | Mitochondria | Ergastoplasm |
| Controls | 1·64±0·2 | 1·52±0·1 | 1 |
| Tests | 1·19±0·1 | 1·47±0·3 | 1 |

We observed that with the method selected by us of seperating the fractions in the irradiated coleoptiles, the weight ratio of protein in the nuclear fraction fell by 31 per cent, the protein amount of the ergastoplasm fraction fell by 27 per cent and the protein content in the mitochondrial fraction increased 1·7 times (Table 5).

TABLE 5. PROTEIN CONTENT IN MG DRY WEIGHT IN ISOLATED CELL FRACTIONS OF IRRADIATED AND UNIRRADIATED COLEOPTILES (AVERAGE DATA FOR THREE EXPERIMENTS)

| Samples | Fraction | | |
|---|---|---|---|
| | Nuclei | Mitochondria | Ergastoplasm |
| Controls | 8·9±0·7 | 26·9±1·0 | 43·0±1·1 |
| Tests | 6·2±0·4 | 47·3±1·1 | 31·9±3·0 |

Together with reduction in the weight of the nuclear fract on there was a fall in the number of nuclei in the irradiated coleoptiles. The number of nuclei was determined by direct count in the homogenate before fractionation and also in the purified nuclear fraction. Thus, the homogenate of 15 g moist coleoptiles before fractionation contained $(225\pm15)\times10^6$ nuclei in the controls and $(116\pm11)\times10^6$ nuclei in the case of irradiation. Consequently, in the irradiated plant the number of nuclei was half that in the unir-

TABLE 6. NUMBER OF NUCLEI IN WHOLE HOMOGENATE AND IN PURIFIED NUCLEAR FRACTION OBTAINED FROM UNIFORM MOIST MASS (4 G) OF IRRADIATED AND UNIRRADIATED COLEOPTILES

| Samples | Experiment No. | Number of nuclei in whole homogenate | Number of nuclei in purified nuclear fraction | Percentage residual nuclei |
|---|---|---|---|---|
| Controls | 1 | $47{\cdot}04\times10^6$ | $12{\cdot}71\times10^6$ | 27 |
| | 2 | $56{\cdot}28\times10^6$ | $14{\cdot}76\times10^6$ | 26 |
| | 3 | $51{\cdot}66\times10^6$ | $13{\cdot}44\times10^6$ | 25 |
| | 4 | $44{\cdot}52\times10^6$ | $10{\cdot}45\times10^6$ | 25 |
| Tests | 1 | $23{\cdot}02\times10^6$ | $5{\cdot}25\times10^6$ | 23 |
| | 2 | $27{\cdot}41\times10^6$ | $6{\cdot}74\times10^6$ | 25 |
| | 3 | $25{\cdot}32\times10^6$ | $6{\cdot}56\times10^6$ | 26 |
| | 4 | $20{\cdot}91\times10^6$ | $5{\cdot}48\times10^6$ | 27 |

radiated, which probably is the result of the arrest of cell division after irradiation. The losses on purification of the nuclear fraction were uniform in both the controls and tests (as shown by Table 6).

Knowing the normal number of nuclei and that after irradiation in the original homogenate and the protein weight in the fractions we estimated the amount of protein of the fraction per cell (we assume that the number of nuclei is equal to the number of cells). The results do not have absolute values (we do not know the percentage of isolated mitochondria and ergastoplasm), but their ratio apparently reflects the actual ratios if we assume that isolation is similar in the tests and controls as indicated by our counts of nuclei. The following relations presented in Table 7 were obtained on the basis of these premises.

TABLE 7. NOMINAL PROTEIN CONTENT OF ISOLATED CELL FRACTIONS PER SINGLE CELL (IN MG)

| Samples | Fractions | | |
|---|---|---|---|
| | Nuclei | Mitochondria | Ergastoplasm |
| Controls | $0{\cdot}039\times10^{-6}$ | $0{\cdot}119\times10^{-6}$ | $0{\cdot}191\times10^{-6}$ |
| Tests | $0{\cdot}053\times10^{-6}$ | $0{\cdot}407\times10^{-6}$ | $0{\cdot}275\times10^{-6}$ |
| Test/Controls | 1·3* | 3·4 | 1·4* |

* This increase may only be an apparent one since it may lie within the limits of experimental error.

It may be concluded from the results that against a background of insignificant changes, as judged from the incorporation $^{35}S$ methionine into the proteins of the fractions of the irradiated plants (Table 3), the content by weight of protein in the nuclear and ergastoplasm fractions increased slightly and in the mitochondrial fraction several times, which indicates disturbance in protein metabolism in the cell of the irradiated plants.

## DISCUSSION OF RESULTS

In the work [5–6] done on the animal organism, labelled amino-acids were used to characterize the disturbances arising from ionizing radiation in the protein metabolism of the cell fractions, the results being expressed in counts/min/mg substance (protein).

In the present work the indicator of change in protein metabolism of the cell fractions of the plant organism was sulphur labelled methionine and the results are also expressed in counts/min/mg protein. Our findings show that 48 hr after irradiation with $^{60}Co$ $\gamma$-rays at a dose of 10,000 r incorporation of $^{35}S$ methionine into the proteins of the nuclear fraction and the ergastoplasm fraction was almost unchanged and the e was a slightly increased uptake by the proteins of the mitochondrial fraction, while the incorporation of $^{35}S$ methionine into the total protein of the coleoptiles was not disturbed. This conclusion is in good agreement with those from the papers quoted [5, 6]. However, on the basis of these findings it is difficult to explain the considerable changes in the plant organism as indicated by our other findings, namely: depression

of growth of the seedlings by 23·7 per cent, 50 per cent decrease in number of nuclei, 50 per cent reduction in DNA content in the nuclei, reduction in protein content in nuclear fraction (by 30 per cent) and in ergastoplasm fraction (by 33 per cent) and 2-fold increase in protein content in the mitochondrial fraction.

After computing the relative activities of the proteins of the fractions of the irradiated and unirradiated coleoptiles, we found that in the normal the highest relative protein activity is displayed by the nuclear fraction while in the irradiated coleoptiles the highest relative protein activity was observed in the mitochondrial fraction. These findings show the degree of participation of each fraction in the protein metabolism of the cell and correspond to the real profound disturbances in the functional and morphological activity of the irradiated cell discussed above.

D sturbance in protein metabolism in the cell structures after irradiation is demonstrated even more clearly when the protein content of the fraction per cell is computed (Table 6) namely; in the irradiated cell an increase in protein content is observed in all fractions and a particularly large increase in the mitochondrial fraction.

It may be assumed from our findings that in the mechanism of action of ionizing radiation on protein metabolism of the cell, one of the primary factors is disturbance in balance between the rates of the processes of synthesis and decomposition as a result of the suppression of cell division. This is most strikingly manifest in the mitochondrial fraction where after irradiation the rate of protein synthesis was almost unchanged but the protein content rose 4 times. It is interesting to note that Braun [17] observed change in the structure and enlargement of the mitochondria of the mouse liver 24 hr after a lethal radiation dose.

Probably the irradiated nucleus is incapable of utilizing the metabolites of protein metabolism formed by the mitochondria (therefore we also observed such a tremendous — 4 times — increase in protein in the mitochondria) or the transport of these metabolites is disturbed and possibly atypical proteins are formed not peculiar to the normal cell [18].

## SUMMARY

(1) A modified method of isolation of nuclei was applied to the test object (wheat seedlings).

(2) Forty-eight hours after irradiation with $^{60}Co$ γ-rays at a dose of 10,000 r, incorporation of $^{35}S$ methionine into the total proteins of the stems was not disturbed. At the same time incorporation of $^{35}S$ methionine into the proteins of the cell fractions was changed; the incorporation of $^{35}S$ methionine into the protein of the mitochondrial fraction was somewhat increased with almost no change in this respect for the nuclear and ergastoplasm fractions.

(3) γ-radiation at a dose of 10,000 r led to depression of growth of the seedling by 23·7 per cent and to a decrease in the number of nuclei by half.

(4) In the irradiated cell there was a distinct increase in the protein content in the mitochondrial fraction on calculation per cell.

I wish to express my deep gratitude to Prof. A. M. Kuzin for his careful guidance and valuable advice in carrying out the work.

*Translated by* A. Crozy

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* There is a translation into English of this work.


# EXACT DOSE X-IRRADIATION OF VARIOUS REGIONS OF THE HEAD AND VISUAL SENSATIONS — X-RAY LOCATION METHOD OF STUDY OF THE REACTIVITY OF THE CENTRAL NERVOUS SYSTEM*


G. K. GURTOVOI and YE. O. BURDIANSKAIA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow
and The Helmholz State Research Institute for Diseases of the Eye, Moscow


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PERCEPTION of a flash of light at the moment of irradiation with X-rays is commonly known as X-ray phosphene. In the period from 1895 to 1906 when X-ray phosphene was first studied (and Roentgen himself was the pioneer of this study [1, 2]) due attention was not paid to the problems of radiation dosimetry. Between 1906 and 1932 no experimental work on X-ray phosphene was published—apparently because of the reported harmful effects of X-rays. In 1932, study of X-ray phosphene was renewed

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by two investigators [3, 4] independently of each other. After 1932, papers on X-ray phosphene appeared regularly. In the interval from 1932 to 1954 some workers applied dosimetry but only in relation to the intensity of X-radiation [5].

The first attempt to define the threshold value of dose (and not dose rate) of X-irradiation of the eyeball necessary to perceive a light flash was made in 1954 [6]. However, this work was marred by several serious shortcomings which led us to repeat these published measurements in 1959 [7]. The threshold value of dose was determined from work on the human electroretinogram [8].

This research was concerned with irradiation of the eyeball. Attempts have been made to investigate the reactivity of the nerve stump, after removal of the eyeball, and the brain on the basis of a study of X-ray phosphene. However, the investigations known to us are of a qualitative, tentative character and did not employ definite doses of X-irradiation [9].

VOL. 5 1960

It seemed to us that a study of the reactivity to X-radiation of the various parts of the central nervous system could be successfully undertaken by irradiation of the various parts of the head with known doses. Consequently, the question is one primarily of the application of an X-ray location technique and secondly, measurement of dose and not dose rate.

## 1. APPARATUS AND METHODS

The apparatus and details of the methods have been described in our article [7].

The apparatus made possible irradiation of the various regions of the head with low doses of X-radiation of a known and adjustable value.

The near-threshold dose range was determined, i.e. the two doses were found at one of which the subject always perceived a light flash and never did so with the other. The range was determined, as a rule, on the basis of 6–9 irradiations at near-threshold doses.

In contrast to work [7] in this work, we did not use a semi-circular diaphragm since its use is meaningful only for direct irradiation of the retina when the apparent direction of the light flash serves as control of the correctness of the subject's responses. In this work, we used a beam of radiation with a circular cross-section, the diameter of which at the surface of the head was $\approx 35$ mm. The correctness of the subject's responses were controlled as follows; among the irradiations some were blank (X-ray unit was not switched on though the subject was not aware of this).

The experiments were carried out in complete darkness, after dark adaptation for 1 hr (including experiments with completely blind persons). Where necessary, the side of the healthy eye was additionally screened from the scattered X-rays by a special lead screen.

*Quality of radiation*

(A) In the experiments with direct irradiation of the eyeball the quality was characterized by 135 kV and by a copper filter 2–7 mm thick.

(B) Experiments with doses of 5–160 mr were carried out at 135–175 kV with aluminium filters 0·5–3·5 mm thick. In these two cases (A and B) the duration of radiation with a single dose was 0·14 sec.

(C) Experiments in which we used doses of 0·7–3 r were run at 175–210 kV without filter, or with an 0·5 mm aluminium filter; duration of radiation with single dose about 1 sec.

For some cases the absorbed dose given in the summary was estimated (roughly, to an order of magnitude).

## 2. VISUAL SENSATIONS ON THRESHOLD IRRADIATION OF HEALTHY EYE AND THEIR CAUSE

The following ranges were obtained for four normal subjects; the observers always saw the light flash at doses of 0·8–3 mr (different observers — different doses) and never saw it from 0·2 to 1·6 mr. For example, one of the four subjects always saw the light flash at doses of 0·8 mr and never saw one at 0·2 mr; another subject always "saw" 3 mr and never saw 1·6 mr. These results obtained with a round diaphragm are in good agreement with the ranges obtained in our work with a semicircular one [7]; from 1 to 3 mr for seeing and from 0·3 to 0·8 mr for not seeing.

Similar findings on the remaining healthy eye for patients with one eye removed are given in Table 1.

It may be concluded from a number of studies, that at sufficiently large X-radiation doses, the sensation of light develops as a result of excitation of the actual retina. These are studies in which the observer recognized figures in the beam of X-radiation and also those in which the narrow beam of X-radiation intersected the eyeball perpendicular to its optical axis, and the subject perceived two bright spots. However, in this work very large doses of the order of 1r were used.

It is not clear to us whether this conclusion can be extrapolated to threshold doses without additional experiments. It may well be that with reduction in dose to threshold values sensation of light is no longer induced by reactivity of the retina, but by another substrate of the eye, for example, the optic nerve. Solution of this problem, as we shall see, is important for further research.

Analysis of the results of our earlier published experiments [7] shows that even at threshold doses, X-radiation also excites the retina. This conclusion follows from the fact that at threshold irradiation of the left part of the retina, the observer perceived a flash of light on the right, and conversely [7]. Such an interrelation is only possible if the threshold X-radiation excites the region of the retina and not another reactive substrate of the eye, say, the optic nerve.

Hence in subsequent threshold investigations of irradiation of various regions of the head we must consider the retina as a type of X-ray sensitive instrument having a definite, known threshold reactivity.

## 3. VISUAL SENSATIONS ON THRESHOLD IRRADIATION OF THE REGION OF THE REMOVED EYE

The articles [9, 10] quote experiments with X-radiation soon after removal of one eyeball (enucleation). On irradiation of the operated side the authors did not observe visual sensations in the patients, while irradiation of the normal eye gave visual sensations. A similar conclusion was reached by London [11] in experiments with radium.

The authors do not indicate the doses but they were not small as the subject was in the beam for a fairly long time. Probably the doses were of the order of 1r.

Our experiments do not confirm these observations. We investigated six patients after removal of one eyeball for tumour; four females and two males aged from 25 to 60 years. The investigations were made from 8 days to 10 months after enucleation.

The X-ray beam was directed frontally on the region of the removed eye (i.e. perpendicular to the plane of the face).

All the patients without exception perceived the light flash on irradiation of the region of the removed eye with defined doses of X-radiation. Moreover, for each subject the near-threshold dose range could be found. On irradiation of the enucleated region the patients subjectively localized the light flash on the side at which the dose was delivered.

In three patients the effect of X-ray phosphene was also investigated at the same time for the normal eye. In these cases the near-threshold dose range was also established. The results are given in Table 1.

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TABLE 1. NEAR-THRESHOLD DOSE RANGE ON IRRADIATION OF NORMAL EYE AND ENUCLEATED REGION (mr)

| Subject | Time elapsing since operation | Normal eye | | Enucleated region | |
|---|---|---|---|---|---|
| | | Always sees | Never sees | Always sees | Never sees |
| Pl | 8 days | —* | — | 50 | 20 |
| G | 13 ,, | — | — | 50 | 20 |
| Pr | 17 ,, | — | — | 17 | 5 |
| R | 20 ,, | — | — | 55 | 20 |
| D | 7 months | 2·1 | 1·3 | 120 | 90 |
| R | 9 ,, | 1·2 | 0·7 | 150 | 70 |
| I | 10 ,, | 4·1 | 2·8 | 130 | 90 |

* No experiment carried out.

Why was it that the previous investigators did not detect X-ray phosphene on irradiation of the enucleated region? As stated above, these experiments are quoted in papers [9, 10]. We were unable to discover in the Moscow libraries the journals containing the quoted papers which go back to 1897 and 1898 and it is therefore difficult for us to judge the reasons for the absence of X-ray phosphene in the experiments of these other authors. The most probable explanation is that the experiments were conducted with a normal eye completely unadapted or poorly adapted to the dark another possible factor is that controls of the reactions of the subject were not set up with sufficient care.

## 4. CAUSE OF VISUAL SENSATIONS ON THRESHOLD IRRADIATION OF THE REGION OF THE REMOVED EYE

In our view, the cause of the phenomenon resides in the reactivity of the retina of the remaining healthy eye to scattered X-rays. We shall demonstrate this by refuting other possible suppositions.

(A) One possible assumption of the cause is the reactivity of the stump of the optic nerve remaining after removal of the eyeball. This seems plausible for the following reasons: (1) because of the subjective sensations of the subject (it seems to him that the light flash is localized on the side at which the irradiation is delivered); and (2) because of the increase in the threshold on passage from the normal eye to the enucleated region (Table 1). However, the following two factors — each in itself — discount this assumption.

Firstly, if the cause really was to be found in the reactivity of the stump of the optic nerve, then passage from irradiation of the stump to irradiation of regions adjacent to the enucleated one ought to result in a sharp rise in the threshold dose, or even to the absence of X-ray phosphene. We carried out experiments in which as well as irradiation of the enucleated region, the tip of the nose and the middle of the forehead were irradiated. The results are given in Table 2. The Table shows that there was no sharp rise in the near-threshold dose range on removal of the beam of radiation from the nerve stump; moreover, in a number of cases there was even a reduction.

TABLE 2. NEAR-THRESHOLD DOSE RANGE ON IRRADIATION OF ENUCLEATED REGION AND REGIONS OF MIDDLE OF FOREHEAD AND TIP OF NOSE (mr)

| Subject | Time elapsing after operation | Enucleated region | | Middle of forehead | | Tip of nose | |
|---|---|---|---|---|---|---|---|
| | | Always sees | Never sees | Always sees | Never sees | Always sees | Never sees |
| Pl | 8 days | 50 | 20 | 70 | 20 | — * | — |
| G | 13 ,, | 50 | 20 | 70 | 20 | 60 | 20 |
| D | 7 months | 120 | 90 | 80 | 60 | 60 | 40 |
| R | 9 ,, | 150 | 70 | 160 | 60 | 130 | 60 |
| I | 10 ,, | 130 | 90 | 150 | 70 | 100 | 70 |

* No experiment carried out.

Secondly, if the cause was the reactivity of the optic nerve stump, then the threshold dose for X-ray phosphene would be intimately related to the time elapsing after enucleation. It is well known that after removal of the eyeball the remaining segment of the optic nerve atrophies after a few weeks and, in any event, after 7 months. Therefore, if the assumption were true, in Table 1 the threshold doses for the last three patients (time elapsing after enucleation 7–10 months) would be much greater than for the first four (time elapsing after enucleation 8–20 days); for the last three patients X-ray phosphene might even have been absent. In the Table such a pattern is not found. The insignificant increase in the threshold doses for the last three in relation to the first four patients may be attributed to the after-effects of removal of the eye; changes occurring in the post-operative period might have affected the reactivity of the normal eye and the degree of scatter of the X-rays.

Incidentally, we would note that in Table 1 a quite marked difference in the threshold doses for various patients (up to 10 times) is observed. This difference may be explained by the individual features for scatter of the X-rays in the various patients. Another

factor might have been that the different patients might have chosen different criteria for saying whether they had already perceived the flash of light after delivery of the dose (they might say "I see" with a greater or lesser degree of confidence).

Thus, the results of the experiments indicate that the cause of X-ray phosphene on irradiation of the enucleated region is not the reactivity of the optic nerve stump. However, these results become intelligible if we assume as the cause the effect of scatter of X-radiation on the retina of the normal eye.

(B) But if we reason from the experiments described under (A) we may assume that the sensation of light might have been due to the reactivity of certain parts of the brain irradiated in all three cases (i.e. on localization of the beam on the enucleated orbit, the middle of the forehead or the tip of the nose) by direct rays.

This assumption was checked experimentally. The experiment was so performed that, in one case, direct X-radiation impinged on the brain, and in the other, did not. If direct irradiation of the brain is immaterial then both directions of the beam should have given roughly uniform ranges; if direct irradiation is decisive, then the ranges should have varied sharply. The experiments with the patients submitted to enucleation present some psychological difficulties, therefore we carried out this investigation on persons with two normal eyes.

The regions of the middle of the forehead and nose tip were irradiated with the the beam directed frontally, i.e. perpendicular to the plane of the face, and for comparison purposes, the region of the cheek with the beam perpendicular to the sagittal plane, i.e. parallel to the plane of the face. In the first two cases the distance of the beam from the eyes was equal; in the last case, the minimal distance of the beam from the eyes was equal to the distance of the beam from the eye in the first two cases. Three persons with normal eyes took part in the experiments; two men and one woman, aged from 20–50 years.

TABLE 3. NEAR-THRESHOLD DOSE RANGE FOR NORMAL SUBJECTS ON IRRADIATION OF REGIONS OF MIDDLE OF FOREHEAD, TIP OF NOSE AND CHEEK (mr)

| Subject | Middle of forehead | | Tip of nose | | Cheek | |
|---|---|---|---|---|---|---|
| | Always sees | Never sees | Always sees | Never sees | Always sees | Never sees |
| M | 100 | 30 | 120 | 40 | 120 | 40 |
| V.Iu | 30 | 10 | 30 | 10 | 50 | 30 |
| V.E | 30 | 10 | 50 | 30 | 50 | 30 |

The results of the experiments are given in Table 3. They show that the cause of X-ray phosphene in our case is not irradiation of certain parts of the brain. The results are not inconsistent with the hypothesis on the effect of scattered X-radiation on the retina.

(C) Finally, it could be assumed that in the experiments described under section (A), the subject responded to irradiation of the nerve endings in the skin. Some grounds for this assumption can be seen in the fact that in these experiments we were unable

to ascertain directly whether the subject actually saw the light flash, or whether he took, some other sensation as a light flash?

This assumption can be quite categorically rejected merely from a comparison of the data for the normal eye (Table 1) and the results of irradiation of other regions of the face (Tables 2 and 3). In fact, irradiation of the normal eye does not involve a larger number of skin receptors than irradiation of other parts of the face; however, the threshold on irradiation of the normal eye was considerably lower than in other cases.

This assumption is also disproved by special experiments in which the finger tips with their copious supply of skin receptors were irradiated. Two subjects (V. B. and V. E.) did not distinguish a dose of radiation of 2 r from mock radiation (the X-ray unit was not swit hed on, though the subject did not know this). The third subject (M) was given a dose of 0·7 r which he also did not perceive.

Perhaps the strongest argument against this assumption is provided by the negative experiments with completely blind persons which are described later on. The blind did not perceive a dose of up to 3 r in these experiments.

(D) in sections (A), (B) and (C), various assumptions on the cause of the origin of visual sensations on threshold irradiation of the region of the enucleated eye are dismissed.

For reasons similar to those above we can rule out the assumption that the subjects reacted to irradiation of the facial nerves, e.g. the trigeminal nerve, and other similar assumptions. One possible cause remains — the reactivity of the retina of the normal eye to the scattered X-rays. This factor explains the results of all the experiments described in Tables 1–3 and under sections (A), (B) and (C).

The fact that the subjects localized the flash of light on he side of the removed eye must be ascribed to subjective factors; the experiment was so performed that the subjects knew from which side radiation was being delivered.*

It was of interest to check our assumption by comparing the coefficients of scatter obtained in experiments on man and on models.

For man, he scatter coefficient was computed from experiments on three enucleated persons. The threshold dose taken was in the middle of the range. The numera or of the coefficient fo each subject — threshold doses for the normal eye — was determined from Table 1; the denominator by averaging the thresholds for the same subject obtained from Table 2. For the three subjects, the following scatter coefficients were obtained: 0·7, 2·3 and 3·4 per cent. The averaged coefficient was 2·1 per cent.

The experiments with models were carried out in water and on a skull immersed in water; of course, the distance of the beams to the dosimeter chamber (6–7 cm) was equal to the corresponding distance in experiments on persons with a removed eye. In three experiments the coefficients 0·4, 0·7 and 0·8 per cent were obtained. The averaged coefficient of scatter was 0·6 per cent.

* This fact might be investigated in greater detail with different design of experiment — namely — subject unaware of which region of the face is being irradiated.

If we compare the individual coefficients in the series 0·7, 2·3 and 3·4 per cent and the series 0·4, 0·7 and 0·8 per cent there is no significant difference between them. The difference between the two series of coefficients becomes understandable if we bear in mind the highly divergent conditions of their determination. Firstly, the differences in the receptors: the dosimeter chamber (2 $cm^3$) and the retina of the human eye; and secondly, the differences in the scattering media.

## 5. VISUAL SENSATIONS ON IRRADIATION OF OCCIPUT

The occipital regions of the brain were irradiated; the beam was perpendicular to the sagittal plane so that no direct rays impinged on the eye. Four subjects with both eyes normal took part in the experiment.

Two of them did not differentiate a dose of 1–1·5 r from mock radiation; the dose was delivered repeatedly. The third subject sometimes saw and sometimes did not see a dose of about 150 mr (this subject was given a larger dose). The fourth subject always saw the dose of 150 mr and never saw a dose of 90 mr.

The causes of X-ray phosphene on irradiation of the occiptal regions of the brain may be both their reactivity and visual sensations because of the effect of scattered light on the retina. This problem requires further study.

## 6. EXPERIMENTS WITH COMPLETELY BLIND PERSONS

Five males aged from 22–37 years and blind in both eyes were investigated. In these persons complete loss of vision in both eyes, together with destruction of the retina, had occurred 16–25 years ago for various reasons. In the experiments, the beam of rays was directed frontally: either through the orbit with the head in the usual position or through the tip of the nose with the head raised so that the beam intersected the chiasma. None of these subjects could distinguish a dose of 3 r from mock irradiation even on repeated irradiation.

It is known from patho-histological investigations that with peripheral lesions in the visual analyser degenerative changes set in in the visual cor ex after periods measu·ed in several years. It may thus be assumed that in our blind persons (who became blind 16–25 years ago) there were marked degenerative, atrophic changes in the visual cortex.

Thus, after atrophy in the visual tract and cortex stimulation of the cerebral formations with X-radiation at a dose of 3 r did not elicit visual sensations.

## SUMMARY

(1) On frontal irradiation of the normal human eye, the primary substrate excited by threshold doses of X-radiation causing perception of a light flash, is the retinal region. The threshold dose for the retina is about 1 mr; the threshold absorbed dose is about 1 mrad.

(2) Persons with a removed eyeball on irradiation of the operated region with a frontal X-ray beam perceive a flash of light at definite doses of radiation. Six persons

taking part in the experiment saw a flash at doses of 17–150 mr (different observers saw flash at different doses) and did not see flash at doses of 5–90 mr.

(3) The cause of X-ray phosphene on frontal irradiation of the region of the removed eye with threshold doses (17–150 mr) is neither the reactivity of the optic nerve stump, nor the reactivity of the parts of the brain irradiated, nor the sensitivity of the skin receptors. In the cases considered, the cause of X-ray phosphene was irradiation of the retina of the normal eye by scattered X rays. The averaged coefficient of scatter was about 2 per cent.

(4) On irradiation of the occiptal regions of the brain in subjects with normal eyes at a dose of about 150 mr, one subject perceived a flash of light. In this case, the absorbed dose for the occipital regions of the brain was about 40 mrad. The reason for this phenomenon must be explored.

(5) Stimulation of the cerebral formations (after atrophic changes in the visual tract and cortex) by X-radiation with a dose of up to 3 r, did not cause visual sensations. With our disposition of the beam, the absorbed dose for the chiasma was about 1 rad and for the occipital regions about 0·2 rad.

(6) In the study of threshold visual sensation and their causes on X-irradiation of various regions of the head it is important to apply defined doses (not dose rates) of radiation. Scatter of the X-rays in the head must be taken nto consideration.

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*Translated by* A. CROZY

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# EFFECT OF γ-RAYS AND NEUTRONS ON DRIED SEEDS OF AGRICULTURAL PLANTS*

S. A. VALEVA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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IT is known that air dried plant seeds, not only of different species but also of different varieties, vary in radiosensitivity, as seen in inhibition of growth and survival rate of plants grown from ir adiated seeds [1–4]. It has been shown [3–6] that in inhibition of growth an important rôle is played by damage to the cell nucleus and that nuclei vary in sensitivity to γ-irradiation not only in plants of different varieties but in the different tissues of one and the same plant. The reason for this variable radiosensitivity of nuclei with an equal number of chromosomes of identical shape and size, still remains unknown. Apparently, the variable sensitivity of plants to radiations is due to differences in the biochemistry and physiology of plant cells and the presence of certain substances, or whole systems, protecting the nucleus from damage. This defence may come into play either during irradiation, via neutralization of the direct effect and the chemical after-effect of radiation, or in the period of transition of the sced from a resting to an active state during germination and so contributed to the restoration of the destroyed st uctures. What are these protective systems or substances, what is their mechanism of action and which cell systems are responsible for the damage ? An approach to a solution of these problems is to study the effect of ionizing radiations on closely related plants of different varieties of the same species or even to lines of the same variety. Such plants are similar in their biochemistry and physiology, but may differ considerably in their reaction to irradiation.

In this respect it is relevant to study the sensitivity of such plants to radiations of different linear ionization density. It is known that the effect of radiations with a low specific ionization density is greatly modified by a numbêr of factors such as: humidity, oxygen, temperature, etc. but that these factors have little influence on the efficacy of radiations with high specific ionization density.

We shall attempt to explain from various angles the reasons for the variable radiosensitivity of plants by studying reactions of closely related plants. This paper reports on part of this work.

## METHODS

The study was concerned with two varieties of maize — Podmoskovnaia and Nemchinovskaia — the self-pollinated lines of maize VIR-44 and VIR-38 and a hybrid between them. The findings of laboratory experiments and cytological investigations

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were utilized. In addition, reference was made to certain findings from observations on growth and development of 11 varieties of beans and ten varieties of peas in field and laboratory conditions. In all cases air dried seeds were irradiated.

Irradiation with γ-rays was carried out from a $^{60}Co$ unit with a dose rate of 600 r/min. For neutron irradiation we used reactor fission neutrons, the flux of which was about $2\times10$ neutrons/cm$^2$/sec. The dose of γ-radiation (with filtration through lead) was about 30 per cent of the dose produced by the neutrons. The thermal neutrons were cut out with B4-C3 filters

The seeds after irradiation were germinated in Petri dishes and the seedlings were transplanted to polymethyl methacrylate meshes placed on jars containing tap water. The measurements were made on the 14th day after germination of the seeds. The height of the seedlings and the length of the roots were measured. For cytological investigation the rootlets were fixed during the first mitosis and aceto-carmine squashes were prepared. The number of normal cells and cells with bridges and fragments in the anaphase and telophase stages was estimated.

## RESULTS

(1) Study of the sensitivity of γ-rays of self-pollinated lines and their hybrid shows (Table 1) that the VIR-44 line was more resistant than the VIR-38 line, however, the greatest resistance to γ-radiation was displayed by the hybrid between these lines showing heterosis.

TABLE 1. RADIOSENSITIVITY TO γ-RAYS OF SELF-POLLINATED LINES OF MAIZE AND THEIR HETEROTIC HYBRID

| Dose (r) | VIR 38 | | VIR 44 | | VIR 44×VIR 38 | |
|---|---|---|---|---|---|---|
| | Height of seedlings (cm) | Percentage chromosome transformations | Height of seedlings (cm) | Percentage chromosome transformations | Height of seedlings (cm) | Percentage chromosome transformations |
| Controls | 28·0±1·3 | | 23·0±1·0 | | 28 6±1·1 | |
| 12,000 | 5·1±1·1 | 85·0±1·9* | 16·2±1·1 | 54·5±2·6 | 17·9±1·1 | 49·2±2·7 |
| 17,000 | 2·7±0·7 | | 12·0±1·0 | | 13·6±1·2 | |

* Cytological analysis made only for the dose of 12,000 r.

In the study of the rootlet cells in the period of the first mitosis it was found that the least damage was sustained by the nuclei of the hybrid and the greatest by the most sensitive line VIR-38. Table 1 shows that there is a positive correlation between the growth inhibition and the number of impaired mitoses as already established for wheat and the pea by Khvostova *et al.* [5, 6].

(2) Table 2 gives the results of irradiation of two varieties of maize — Nemchinovskaia and Podmoskovnaia — by neutrons and γ-rays. The Table shows that judging by all three criteria both varieties differ sharply from each other in relation to both types of radiation. The Nemchinovskaia maize variety was more resistant not only to γ-rays but also to neutrons. The difference in radiosensitivity of the varieties was particularly manifest at high radiation doses.

TABLE 2. SENSITIVITY TO NEUTRONS AND GAMMA RAYS OF TWO VARIETIES OF MAIZE — NEMCHINOVSKAIA AND PODMOSKOVNAIA

| Doses of radiation with neutrons, (rep), and γ-radiation (r) | Nemchinovskaia | | | | | Podmoskovnaia | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Length of leaves 14 days after germination, (cm) | Percentage of controls | Length of rootlets 14 days after germination, (cm) | Percentage of controls | Percentage of ana- and telophase with chromosome trans-formations | Length of leaves 14 days after germination, (cm) | Percentage of controls | Length of rootlets 14 days after germination, (cm) | Percentage of controls | Percentage of ana- and telophase with chromosome trans-formation |
| Controls | 17·5±1·5 | 100 | 22·4±1·8 | 100 | 1·3±0·4 | 18·9±1·1 | 100 | 28.2±2·3 | 100 | 2·5±0·7 |
| 1000 (n) | 14·4±1·0 | 82·2 | 11·8±1·7 | 53·3 | 29·4±2·0 | 13·7±1·0 | 72·1 | 9·6±0·9 | 34·2 | 31·8±2·2 |
| 2000 (n) | 12·2±0·8 | 69·6 | 11·0±0·7 | 49·5 | 53·0±2·9 | 6·6±0·8 | 34·5 | 5·6±0·8 | 19·3 | 69·5±2·5 |
| 3600 (n) | Died | — | — | — | 73·3±2·7 | Died | — | — | — | 83.1±2·9 |
| 12,000 (γ) | 14·6±0·8 | 83·4 | 16·7±1·6 | 75·0 | — | 11.9±1·1 | 62·6 | 11·2±1·2 | 39·5 | — |
| 17,000 (γ) | 11·6±0·9 | 68·0 | 13·5±1·7 | 60·8 | 65·0±2·5 | 6·6±0·9 | 34·7 | 4·8±0·1 | 16·9 | 71·5±2·6 |
| 22,000 (γ) | 7·8±0·7 | 44·6 | 8·0±1·0 | 36·2 | — | Died | — | — | — | — |

(3) The observations on growth and development in laboratory and field conditions of 11 varieties of bean and ten varieties of peal differing in sensitivity to γ-rays showed that the radio resistant varieties, as compared with the radiosensitive ones, were distinguished by a greater degree of germination, more intensive growth (especially in the first period of life) and accelerated development, which was usually combined with early ripening of the varieties. The control (unirradiated) plants of the radioresistant varieties more rapidly passed from the cotyledon phase to the phase of the first two first genuine leaves i.e. more rapidly passed through the stage in which the greatest destruction of the seedlings was observed in the irradiated plants. The plants with greater regeneration powers survived, i.e. those with greater energy of cell division. The results of study of four bean varieties are shown in Table 3 as an example.

## DISCUSSION OF RESULTS

The observations reported on the growth and development of radioresistant and radiosensitive varieties show that resistance to irradiation is associated with the ability of the plants to show intensive growth and development especially in the first period of life.

Thus, study of the radiosensitivity of the self-pollinated lines of maize and their heterotic hybrids showed the latter (with greater growth powers and stronger development) to be more resistant (Table 2). A similar picture has been found for maize [7] and mice [8, 9]; it was found that heterosis similarly affected the rise in their resistance to radiation.

A possible explanation of these facts is that in the organisms with greater growth energy, the regeneration of tissues is more rapid; the undamaged cells (with undisturbed chromosome balance) more rapidly replace the damaged dying ones. Our observations show that in hybrids and also lines and varieties more resistant to radiations, the first mitosis occurs earlier than in the sensitive forms. The mitotic activity in the meristem of the rootlets in the resistant varieties is also higher.

Study of the rootlet cells in the period of the first mitosis showed that in the resistant plants the nuclei had undergone less damage.

Obviously, the same systems, apparently, the energy systems of the cell, prevent chromosome disturbances, and at the same time intensify the mitotic activity of the nuclei. Of interest in this connexion is the fact discovered in our laboratory that the RNA content in the rootlet meristem of the radioresistant pea variety is greater than that for the sensitive variety. As is known, RNA may be one such energy system, but this should not be taken to imply that even in this particular case RNA is solely responsible for the defence mechanism.

It has been established by Swedish investigators [10] that two pea varieties differing in sensitivity to γ-radiation respond identically to irradiation with fast neutrons. These workers consider that this is due to the great influence exerted by environmental factors (oxygen, humidtiy, temperature) on the effects of radiations with low specific ionization (X- and γ-rays) and to the slight effect of these factors on the action of neutrons.

TABLE 3. INDICES OF GROWTH AND DEVELOPMENT FOR RADIORESISTANT AND RADIOSENSITIVE BEAN VARIETIES

| Name of variety | Day of appearance of complete shoots after sowing | Percentage germination of plants | Day of appearance of first two genuine leaves in 50 per cent plants | Height of plants 56 days after sprouting | Percentage surviving plants at end of vegetation |
|---|---|---|---|---|---|
| Controls | | | | | |
| Sviatoi Andrei | 13 | 97·3 | 17 | 38·2±0·4 | 96·7 |
| Krasnyi Rubchik | 15 | 98·5 | 17 | 36·9±0·7 | 98·3 |
| Tyuchadze | 18 | 87·1 | 25 | 28·3±0·3 | 85·8 |
| Robiust | 21 | 68·9 | 28 | 23·8±0·1 | 68·4 |
| γ-irradiation at dose of 10,000 r | | | | | |
| Sviatoi Andrei | 21 | 94·8 | 27 | 23·2±0·6 | 89·9 |
| Krasnyi Rubchik | 21 | 96·9 | 29 | 24·0±0·7 | 94·5 |
| Tyuchadze | 28 | 71·7 | 47 | 15·4±1·2 | 53·8 |
| Robiust | 31 | 43·4 | 53 | 8·6±2·2 | 12·5 |

Note: Height of plants measured from root collar to tip of terminal leaf.

The experiment carried out by us with fast neutron irradiation of maize varieties with variable sensitivity to the γ-radiations gave a different result: the maize varieties differed in their reaction to irradiation with the same dose of neutrons. It is known that sensitivity to neutrons in plants depends on the number and shape of the chromosomes. Polyploid plants and also plants with fine chromosomes are less sensitive to neutron irradiation [10, 11]. However, in our case the plants were of the same species with the same chromosome number and shape. Apparently, in this case, in the radioresistant maize variety there are certain substances giving protection from both types of radiation.

## SUMMARY

(1) The difference in radiosensitivity of self-pollinated lines of maize and their hybrid, is due not only to the variable powers of regeneration of their tissues, but also to the variable degree of susceptibility to damage of the cell nuclei.

(2) The difference in sensitivity to irradiation with neutrons of varieties of the same crop with a uniform number of chromosomes of uniform size and shape, suggests that in the radioresistant variety there is a certain defence mechanism against the effect of radiations both with low and high specific ionization density.

*Translated by* A. CROZY

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* There is a translation into English of this work.

VOL
5
196

## METHODS AND INSTRUMENTS

# SOME IMPROVEMENTS IN THE UEM-100 ELECTRON MICROSCOPE FOR THE INVESTIGATION OF BIOLOGICAL SPECIMENS*

V. L. BOROVIAGIN and M. G. KUPTSOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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THE need to use microscopes with high resolution in investigation of the submicroscopic intracellular organization of biological specimens is obvious [1, 2]. Preparations are now under way in industry to issue a number of instruments of medium [3, 4] and high [5] resolution. However, instruments of the UEM-100 type already available in the laboratory require improvement in the quality. For this purpose we made a number of refinements to the UEM-100 electron microscope to improve the instrument.

The resolution of an electron microscope is determined in the last resort by the parameters of of stabilization of the accelerating voltage, the degree of stabilization of the power supply to the lenses [6], the properties of the complex objective lens [7] and the system of illumination [8], determined by the various aberrations arising from the asymmetry of the magnetic fields. In addition, the quality of the image of the object on the end screen is greatly influenced by the cleanliness of the internal surfaces of the instrument and the presence of external electromagnetic induction [5].

Taking into account these factors influencing the operational quality and the degree of resolution of the instrument we carried out definite improrements in this direction.

(1) Since the instability of the current in the lenses in our instrument (issued in 1957) is close to the rated value (0·0012–14 per cent) [6] and the instability of the high voltage is of the order of 0·05–0·06 per cent, it was naturally necessary to improve the stabilization of the latter by introducing into the supply circuit a high-voltage stabilizer unit of the SN-4 type (power 1 kW, stabilization coefficient 0·05–0·01 per cent) making it possible to supply to the high voltage transformer the stabilized voltage with instability of the order of 0·005 per cent.

* Biofizika **5:** No. 3, 366–368, 1960.

(2) The surfaces of the parts forming the inner trunk of the column of the microscope were polished.

(3) The supply circuit of the lens unit was taken out of the column stand and the oil vapour pump heater was screened, since the electromagnetic fields produced by these may have an undesirable effect on the stability of the images during photography.

(4) A device was designed for centering the diaphragm of the condenser lens with respect to the optical axis of the system without disturbing the vacuum by means of external adjusting screws on the column surface. The criterion for judging the central position of the diaphragm is the absence of displacement of the image of the pencil of electrons on the end screen on both sides of the central position of the beam image on defocusing, with the rheostat of the condenser.

VOL.
5
1960

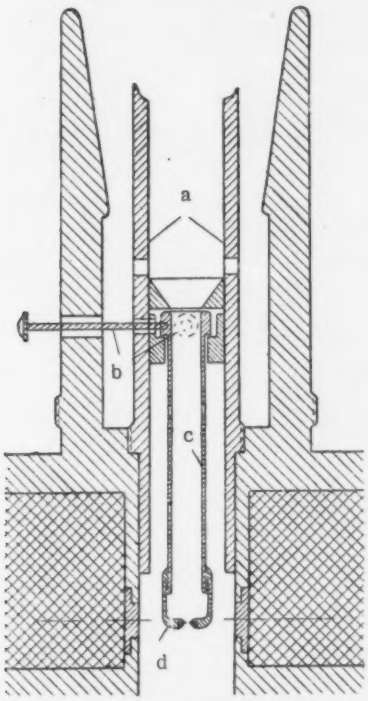


FIG. 1. Design of adjustable illuminating diaphragm. (a) anode cylinder; (b) screws for centering illuminating diaphragm; (c) cylinder of diaphragm; (d) head bearing diaphragm and moving it in vertical direction.

The device depicted in Fig. 1 made of non-magnetic bronze, is fixed in the anode cylinder of the condenser unit and makes horizontal movements of the diaphragm possible. The accurately centred position of the diaphragm at the centre of symmetry of the field determines the minimal aberrations of the condenser lens and the optimum aperture angle of the illuminator (for a given diameter of the condenser diaphragm) which affects the degree of resolution of the given electron-optical system.

In their theoretical calculations, Leisegang *et al.* [6, 7] showed that to reduce the permissible distance it is advantageous to work at small aperture angles of the illuminating system. However, in practice, reduction in the aperture angle of the illuminating system is limited to certain values depending on getting sufficient brightness of the image on the end screen at maximal magnification of the system. In our experiments, such an optimum value for the UEM-100 unit with an accelerating voltage of 60 kV

was a diaphragm of diameter 0·25–0·3 mm (operating at over 80 kV was excluded because of the increase in high voltage pulsation) [5].

(5) Increase in the efficiency of the illuminating system [10],

$$g = \frac{i_B}{i_{IS}}$$

(where $i_B$, the beam current involved in forming the image of the object, and $i_B$, the current of the illuminating system) can be achieved by placing the hot cathode at the level of the lower section and by reducing to 1 mm the Venelt aperture by the addition of a suitable bias resistor (40–70 MΩ). To reduce the area of irradiation of the object and also to reduce the chromatic aberration of the electron source we used as hot cathode a tungsten point welded on the conventional V-shaped hot cathode [11].

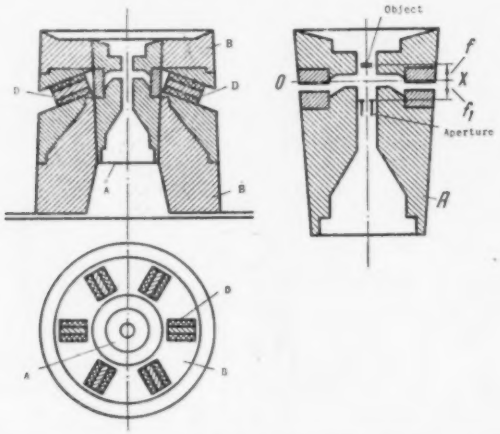


FIG. 2. Design of electromagnetic stigmatizing device. (A) terminal of objective lens; (OX) horizontal axis of symmetry of terminal; (f, $f_1$) distance from object and from aperture diaphragm to line OX; (B) holder of terminal of objective lens; (D) electromagnetic coils with permalloy cores.

On the conventional hot cathode a segment of tungsten wire of the same section is welded strictly vertically to the apex by the method of spot welding. Then at a distance of 0·6–0·8 mm from the site of welding, the welded segment surface is scoured by NaOH solution (concentration selected experimentally) at a constant voltage of 30–50 V. The cone shaped end of the filament so obtained serves as a point source of electron emission. It should also be noted that application of such a hot cathode device produces a considerable increase in the sensitivity of the microscope to different types of vibration, which naturally affects the quality of the photographs. The exclusion of any type of damping and use of an insulated base under the microscope produces conditions for the effective utilization of the hot needle cathode.

(6) The disposition of the objective diaphragm at a distance $f = f_1$ (Fig. 2) from the axis of the slit of the objective lens and the observance of the strict geometry of a circle of diameter 25–35 μ for the diaphragm ensures sufficient contrast of the image (the image of the aperture diaphragm should occupy 80 per cent of the intermediate screen of the projection unit).

(7) In observing these conditions it is necessary to pay attention to the correction of axial astigmatism arising between the pole faces of the objective lens owing to the divergence of the magnetic field from axial symmetry (due to mechanical asymmetry of the components, the magnetic inhomogeneity of the material, etc.), and to temporary astigmatism produced by the asymmetrical charging of the diaphragm and other components of the objective lens.

VOL.
5
1960

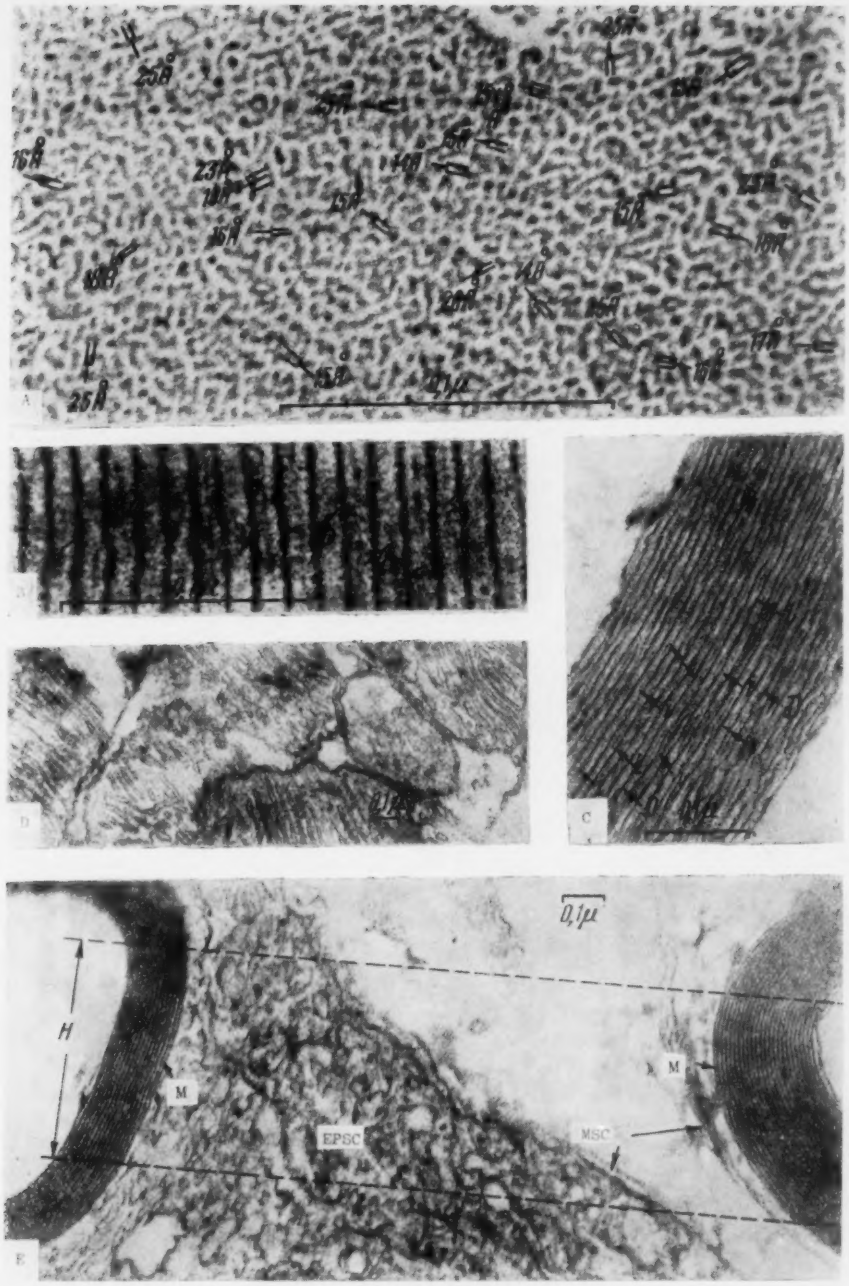


FIG. 3. (A) preparation of platinum and iridium particles. ×600,000 e/o magn. ×40,000 particles with a distance of 16–20 Å resolved; (B) cross-section of myelin fibre of frog sciatic nerve, D-lines are macromolecular lipo-protein chains (25–30 Å); (i) lines indicate osmium impregnation of hydrophobic bonds of phospholipid chains (15–18 Å), ×300,000 e/o magnification ×30,000; (C) same specimen, ×190,000 e/o magnification ×14,000; (E) section of nerve tissue; (D) ultra-fine section through mitochondria in rod of frog retina, internal double membranes visible, ×45,000 e/o magnification 17,000.

To remove the phenomenon of astigmatism, we devised an electromagnetic stigmatizing circuit (Fig. 2) consisting of six electromagnetic coils with permalloy cores (prepared by weight with an accuracy of up to $10^{-5}$ g), arranged in the non-magnetic insertion piece of the holder of the objective lens terminal at an angle of 60° to one another. The coil windings were supplied from accumulators, the supply being regulated by independent rheostats on the panel of the stigmatizing device which was fixed to the column of the microscope. Uniform distribution of the mass of the permalloy cores, which compensated for the asymmetry of the magnetic field of our instrument, made it possible to reduce the phenomenon of astigmatism caused by the non-uniformity of the magnetic field even without switching on the supply to the coil windings of the stigmatizing device. The determination of the optimum system of supplying the windings in each given case was elimination of the astigmatism—according to the Fresnel differaction bands [8].

(8) In the regulator for current in the objective lens a constant resistance of 200 Ω was replaced by an equivalent variable resistance, so facilitating the process of focusing the object. On the control panel a button relay-switch for the primary vacuum pump was placed to reduce the vibration of the column at the moment of photographing the object. To reduce contamination of the microscope column, a trap was placed on the oil vapour pump the design of which was as follows; in the first high-vacuum pipe bend, corrugated copper 110–120 mm in length was inserted. The outer surface of the high vacuum pipe was tightly wrapped with copper tubing (diameter 10–8 mm), or a thin-walled rubber tubing (diameter 10–12 mm), over its entire course with an independent water supply.

In high resolution microscopes at low aperture angles of the illuminating system and objective lens the quality of the image is greatly affected by the scatter of electrons by the object itself and by the film bearing the object. Therefore, in work with such microscopes the investigator must pay considerable attention to the quality of the object (its purity, thickness of film, quality of section, etc.). It must be borne in mind that resolution of structures in tissue sections for an accelerating voltage of 60 kV depends on the thickness of the section and is 0·1 of its size [12]. The effect of slipping of the film relative to the objective grid is greatly reduced by using flat or electrolytic grids or pre-treated ordinary net grids [9]. A good result is also given by perforated films fixed by carbon dusting on both sides. Figure 3 (E) shows the effect of the non-uniformity of section thickness (350–450 Å) on the resolution of submicroscopic macromolecular structures of nerve tissue (×70,000, e/o magnification ×14,000, (M) myelin; (MSC) is membrane of Schwann's cell; (EPSC) is ergastoplasm of Schwann's cell).

## SUMMARY

A number of adjustments and refinements to the electron-optical system of the UEM-100 microscope have been made, making it possible to obtain photographs of platino-iridum preparations with a resolution of 16–20 Å (Fig. 3 (A)) against 50–60 Å according to the rated values and of slices of biological specimens, embedded in methacrylate, with a resolution of the order of 25–40 Å (Fig. 3 (B), (C), (D) and (E)).

The current density on the object has been reduced while retaining sufficient brightness of the image on the end screen, this is particularly desirable in investigations of sections of biological objects. A number of adjustments have been made to facilitate the work of the operator.

All the above described improvements may be made with their own resources by any laboratory with an effective apparatus of the UEM-100 type.

In conclusion, it is necessary to express gratitude to Iu. M. Kushnir and G. V. Dershvarts for assistance given in preparing the manuscript for the press.

*Translated by* A. CROZY

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VOL
5
196

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VOL. 5 1960

# METHOD OF FLUORESCENCE MICROSCOPY OF PERIPHERAL BLOOD*

M. Ia. Khodas

State Herzen Oncology Research Institute, Moscow

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In recent years in Soviet radiobiology and clinical radiology there has been a wide development of the fluorescence microscope method of investigating the bone marrow and peripheral blood, interest in which increased after the work of Meisel' and Sondak [1]. Their findings have been confirmed in a number of research institutes.

However, somewhat unexpected were the results of Sergel' and Klimenko [2], Klimenko and Tsybul'skii [3] and also Vaganova [4]. On the basis of their investigations, these workers consider the fluorescence method of peripheral blood investigation not only as the most sensitive laboratory test in studies of radiation pathology, but as a method of prognostic value. Worthy of special mention are the findings of Sergel' and Klimenko on the changes in the fluorescence picture of the blood in the first 30–60 min after single injection into the body, both human and animal, of trace doses of $^{131}I$ and $^{24}Na$.

During 1958 we used the method of fluorescence microscopy to investigate the blood in a number of patients treated radiologically for malignant neoplasms in the Central Oncological Institute.

As fluorescent stain we used acridine orange. The stock solution was prepared by diluting the dye 1:1000 in distilled water and kept in the dark in a tube with a cork stopper wrapped in cellophane. The working solutions were prepared once a week in fresh saline and stored in the same conditions. The blood from the finger was mixed with the fluorescent stain in a leucocyte pipette. The blood was collected up to the first mark, acridine orange added to the second.

The investigations were carried out on a MBI-I microscope with a OI-18 fluorescence attachment and at a magnification of 280 or 400. A T2-N yellow light filter was placed on the eyepiece. Before microscopy the pipette was shaken and a small drop of mixture placed on the slide and covered with a cover slip. A total count of 100 leucocytes was made over different areas and the staining of their nuclei noted (green, yellow, orange, red). To determine more precisely the conclusion on averaging to 100 cells, further investigations were carried out on 36 persons where the calculation of the percentage leucocyte with nuclei stained the different colours was made on a count of 400 cells. The results confirmed the patterns revealed. Statistical treatment shows the reliability of the mean data.

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In order to estimate the degree of constancy of the fluorescent picture of the blood various external factors of the methodological conditions were changed (change in concentration of dye, time of staining, illumination, ambient temperature, etc.).

In 20 normal subjects the peripheral blood was examined with various concentrations of fluorescent stain (1:10,000, 1:13,000, 1:15,000) for which purpose the blood was withdrawn from each person simultaneously in three pipettes. The findings were of uniform pattern and are set out in Table 1.

With a dilution of the fluorescent stain of 1:10,000 the nuclei in 83 per cent (±2·34) of the leucocytes were stained green (limit of variations from 70 to 98 per cent). At a concentration of acridine orange of 1:13,000, the green staining of the nucleus was observed in 92 per cent (±1·48) of the leucocytes (variation from 83 to 100 per cent) and at a concentration of 1:15,000 in 99 per cent (±1·8) of the leucocytes (variation from 96 to 100 per cent).

These results show that the number of leucocytes with nuclei fluorescing green rose, other things being equal, as the concentration of fluorescent stain decreased and that a concentration of 1:15,000 is optimum for evaluating the original data.

TABLE 1. CHANGE IN FLUORESCENCE OF LEUCOCYTE NUCLEI WITH CONCENTRATION OF FLUORESCENT STAIN (COLOUR OF NUCLEUS)

| Name of subject | Concentration of acridine orange | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1:10,000 | | | | 1:13,000 | | | | 1:15,000 | | | |
| | Green | Yellow | Orange | Red | Green | Yellow | Orange | Red | Green | Yellow | Orange | Red |
| V-va | 86 | 13 | — | 1 | 90 | 8 | 2 | — | 100 | — | — | — |
| F-na | 80 | 16 | 4 | — | 93 | 6 | 1 | — | 100 | — | — | — |
| G-man | 92 | 7 | 1 | — | 99 | 1 | — | — | 99 | 1 | — | — |
| G-va | 74 | 21 | 5 | — | 100 | — | — | — | 100 | — | — | — |

In order to check on this relation between the fluorescence of the nuclei and the concentration of fluorescent stain, and on the suitability of these concentrations for investigation of the blood of irradiated humans, we examined 19 patients with malignant neoplasms receiving X-ray therapy (the radiation dose and time of treatment varied).

TABLE 2. STAINING OF LEUCOCYTE NUCLEI IN PATIENTS TREATED RADIOLOGICALLY IN RELATION TO CONCENTRATION OF FLUORESCENT STAIN (COLOUR OF NUCLEUS)

| Name of subject | Concentration of acridine orange | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1:10,000 | | | | 1:15,000 | | | |
| | Green | Yellow | Orange | Red | Green | Yellow | Orange | Red |
| Ts-man | 42 | 19 | 19 | 20 | 98 | 2 | — | — |
| Z-berg | 64 | 18 | 16 | 2 | 98 | 1 | 1 | — |
| S-na | 49 | 36 | 9 | 6 | 100 | — | — | — |
| V-skaia | 71 | 15 | 11 | 3 | 100 | — | — | — |

The results (Table 2) show that in the irradiated patients, as in the normal subjects, the solution of acridine orange with a concentration of 1:15,000 gave green staining of the nuclei of 98–100 per cent of the leucocytes. With this concentration the leucocyte nuclei were never stained red and only in three cases were solitary orange cells noted. In these patients in parallel investigations with the 1:10,000 solution of acridine orange, a sharp decrease in the green cells was usually observed and a correspondingly higher number of yellow, orange and red cells.

Hence, a change merely in the concentration of the fluorescent stain can basically alter the blood picture of fluorescence in the nuclei of the leucocytes to the point of the appearance of nuclei with "pathological" red fluorescence. "Pathological" fluorescence of the leucocytes seen on staining the blood of patients receiving X-ray therapy could not be found with acridine orange in a concentration of 1:15,000.

TABLE 3. RELATION BETWEEN STAINING OF LEUCOCYTE NUCLEI AND TIME OF CONTACT OF BLOOD AND FLUORESCENT STAIN

| Name of subject | Time of staining (min) | Percentage of leucocytes with nuclei of different stain | | | |
|---|---|---|---|---|---|
| | | Green | Yellow | Orange | Red |
| T-va | 1 | 100 | — | — | — |
| (donor) | 15 | 98 | 2 | 1 | — |
| | 30 | 95 | 3·25 | 1·75 | — |
| | 60 | 68·5 | 10·5 | 18·0 | 3 |
| | 90 | 64 | 14 | 17 | 5 |
| | 150 | 52 | 15·75 | 11·25 | 21 |
| S-va | ·1 | 100 | — | — | — |
| (radiation | 15 | 80 | 10 | 5 | 5 |
| therapy for | 30 | 76 | 16 | 4 | 4 |
| malignant | 60 | 66 | 18 | 8 | 8 |
| neoplasm) | 90 | 36 | 34 | 24 | 6 |
| | 150 | 26 | 34 | 22 | 18 |

In eight healthy subjects and two patients with malignant neoplasms receiving X-ray therapy the ratio of the stained cells of the blood taken from each subject in seven pipettes was determined at various (1, 15, 30, 60, 90 and 150 min) intervals of time after taking blood.

It was established that as the time of staining was lengthened, the number of green cells diminished and the number of leucocytes with nuclei stained yellow, orange and red rose progressively (Table 3).

Consequently, the time of contact of blood and fluorescent stain appreciably affects the results of fluorescence microscopic investigation.

According to our results, the optimum time of staining is 30 min from the moment of taking blood, since by then in the normal subject the number of leucocytes with green fluorescence is on average 90 per cent, and in patients undergoing X-ray therapy pathological fluorescence of the nuclei is distinctly manifest (red stain).

TABLE 4. EFFECT OF HEATING MIXTURE OF BLOOD AND FLUORESCENT STAIN ON FLUORESCENCE OF LEUCOCYTE NUCLEI (COLOUR OF NUCLEUS)

| Name of subject | Temperature 18° | | | | Temperature 37° | | | |
|---|---|---|---|---|---|---|---|---|
| | Green | Yellow | Orange | Red | Green | Yellow | Orange | Red |
| V-an | 87·75 | 6·25 | 6 | — | 52·25 | 8·75 | 24·75 | 14·25 |
| L-na | 92·75 | 7·25 | — | — | 68·25 | 8·75 | 9·75 | 13·25 |
| S-ko | 79·5 | 16·5 | 3·75 | 3·75 | 59·0 | 25·5 | 11 | 4·5 |
| K-va | 91·5 | 6·5 | 1·75 | 1·75 | 76 | 5·5 | 11 | 7·5 |

In 20 normal persons a parallel investigation was made of the blood collected in two pipettes. One was kept in diffuse daylight, the other in the dark. It was found that in 19 of the 20, keeping the

pipettes in the light speeded up the staining of the leucocyte nuclei. The number of cells with yellow, orange or red nuclei in the pipettes in the light, was greater than in the parallel samples kept in the dark (on average in the light there were 81 per cent ±1·8 leucocytes with green nuclei and in the dark 90 per cent ±1·8).

Table 5. Effect of cooling mixture of blood and acridine orahge on fluorescence of leucocyte nuclei (colour of nucleus)

| Name of subject | Temperature 18° | | | | Temperature +4° | | | |
|---|---|---|---|---|---|---|---|---|
| | Green | Yellow | Orange | Red | Green | Yellow | Orange | Red |
| V-an | 93·75 | 5·5 | 0·75 | — | 99 | 1 | — | — |
| K-na | 86·75 | 8·0 | 4·75 | 0·5 | 95·25 | 4·5 | 0·25 | — |
| S-ko | 34 | 22 | 28 | 16 | 68 | 8 | 16 | 8 |
| K-va | 52 | 20 | 24 | 4 | 92·0 | 4 | 2 | 2 |

Further, it was noted that illumination of the mixture of dye and blood with ultra-violet and sunlight led to an increase in the number of cells with pathological fluorescence.

These facts show that the action of light accelerates the uptake of fluorescent stain by the blood and changes the fluorescence of the nuclei of the leucocytes from green to yellow, orange and red. Acceleration of staining occurs also on shaking the pipettes containing a mixture of blood and fluorescent stain.

In order to study the effect of the temperature of the surroundings on staining of the leucocyte nuclei, experiments were carried out with a parallel investigation of the same blood from pipettes kept prior to the leucocyte count at temperatures of 18 and 37°. In 12 of 13 cases, heating the pipettes led to a decrease in the number of leucocytes with green nuclei and a corresponding increase in the number of cells with pathological fluorescence (at room temperature on average 93 per cent ±1·55 and on heating 80 per cent ±3·6). The results are given in Table 4. Conversely, cooling (temperature +4°) of the mixture of blood and fluorescent stain retarded staining, for the same staining time, there was a greater percentage of leucocytes retaining green stain of the nuclei than in the sample kept at room temperature (Table 5).

These findings prompted us in subsequent investigations to apply optimum standard conditions in order to obtain more reliable results. Therefore, in an investigation of the peripheral blood of 64 normal subjects (donors), the following conditions were strictly observed: fresh solution of dye, dilution of acridine orange 1:10,000, time of staining of blood (from moment of taking to count) 30 min, pipette containing blood kept in the dark whilso staining at a temperature of 18–20°.

Adhering to these standard conditions we observed the following fluorescent peripheral blood picture: against a background of green plasma, the bright-red platelets were clearly visible, the red cells did not fluorescence; the cytoplasm of the leucocytes was dark green with orange and red inclusions. The nuclei of most leucocytes fluoresced emerald green, the rest yellow, orange and, less often, red. Statistical treatment of the results of the investigations of this group showed that in the great majority of leucocytes (on average 90 per cent ±1·80) the nuclei gave green fluorescence. The maximum number of leucocytes with red nuclei found in the donors was 8 per cent. Red fluorescence of nuclei of solitary leucocytes was found in 25 per cent of the donors (on average 0·6 per cent).

Consequently, by excluding a number of external factors, it is possible to achieve in a fluorescence investigation of the blood of healthy subjects a fairly constant and high percentage of leucocytes with green fluorescence of the nuclei. However, in the standard conditions chosen by us, solitary leucocytes with red nuclei were found even in the blood of healthy subjects.

Our findings clearly show not only the marked variability of fluorescence of leucocytes under the influence of various external factors, but also the definite possibility of enhancing the reliability of the fluorescence of normal leucocytes by observing a standard technique. Ignoring the standard technique,

it is possible in some cases to obtain quite unexpected and inexplicable results when in two simultaneously stained drops of blood of the same person (or experimentally in the same animal) or in two examinations of blood at different times irradiated and not "pathological" leucocytes are found (with red fluorescence of the nuclei) with very wide quantitative variations.

In this respect, it seems to us that the time has come to devize not only a standard but also a unified technique of fluorescent blood microscopy in order to avoid the conflicting and contentious results obtained in various laboratories and institutes. Accordingly a critical scrutiny must be made of any data on "pathological" (red) fluorescence of leucocyte nuclei in the blood of healthy normal subjects at least until reliable "mean" quantitative indices of such fluorescence have been established. The absence of a unified technique of fluorescence microscopy leaves unsolved the problem of pathological fluorescence of nuclei in the blood of patients, in particular, patients with cancer, before irradiation. We consider the point raised in the article by Sergel' and Klimenko to be very debatable; they suggested the possibility of "normalization of leucocytes" (in other words, replacement of pathologically fluorescing cells by normal fluorescing ones) in normal subjects and animals after single injection of trace doses of $^{131}I$ and $^{24}Na$, or after the first therapeutic irradiation in patients. In our investigations of the blood of patients with cancer, on X-ray therapy the phenomenon of "normalization" of the fluorescence of the leucocytes was not observed if all the optimum standard methodological conditions of investigation were respected. It is difficult to understand the phenomenon of "normalization" of fluorescence of leucocytes under the influence of ionizing radiations. Indeed, the authors themselves who first observed this phenomenon do not offer any explanation to account for it.

VOL. 5 1960

## SUMMARY

(1) Increase in the concentration of acridine orange, extension of the time of contact of fluorescent stain and blood, illumination by diffuse light and sunlight, heating and shaking the pipettes lead to more rapid staining of the nuclei of leucocytes with change in the colour of the fluorescence from green to yellow, orange and red.

(2) The application of the technique of fluorescence microscopy requires strict observance of optimum conditions in order to exclude errors. In our experiments these were: dilution of the acridine orange 1:10,000, time of contact of blood and fluorescent stain 30 min, pipettes kept in the dark at constant temperature of 18–20° before counting.

(3) If these conditions are observed on average 90 per cent of the leucocytes of the peripheral blood of donors contain nuclei giving emerald green fluorescence and only 0·6 per cent red.

*Translated by* A. CROZY

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# DETERMINATION OF pH WITH AN ANTIMONY MICROELECTRODE *

G. A. KURELLA and G. A. POPOV

Faculty of Fundamental Biology of the Moscow Lomonosov State University

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THE pH is commonly considered as a function of many important biological processes. In order to determine it in cells and tissues, resort is usually made to methods of staining sections with coloured indicators. This method, however, has a number of serious defects and therefore search for more suitable methods has been in progress for a long time.

Electrochemical methods permit rapid and accurate determination of pH. The use of these methods makes it possible to measure the pH in the cells and tissues not only on one occasion but to follow the course of the pH over a long period of time.

The practice of biopotential measurement with capillary microelectrodes having a diameter from several microns to fractions of a micron has shown that such electrodes do not seriously damage the cells. The results of a number of workers indicate that the electrochemical methods of determining the pH of cells are very promising [1–5]. However, common to all this work, and other studies, is the defect that the relatively large diameter (25 $\mu$ and higher) of the electrodes used so far has placed a serious limitation on the field of application of these methods.

The present work was carried out in order to obtain fine — as small as 1$\mu$ — antimony microelectrodes and to investigate their properties.

The potential of the antimony microelectrode depends on the concentration of antimony oxide on its surface which in turn depends on the concentration of hydrogen ions in the test medium. Chemical equilibrium is almost complete in the first minute. The antimony electrode is usually used in combination with a saturated calomel half-cell, and according to the literature, does not give appreciable salt or colloidal errors [6].

Antimony microelectrodes are prepared as follows: soda-glass tubing with a diameter of about 6 mm and wall thickness of about 0·6 mm is repeatedly drawn over a 3–4 cm length into a capillary of length 6–7 cm in a glass-blowing burner. Then the capillary is cut in two. A small piece of pure antimony weighing about 0·2 g is placed in the semifinished product thus obtained and inserted in the capillary before further narrowing the lumen. Then the capillary is drawn out again on the same burner with a resulting thinning of its walls, and the fused antimony is partially drawn into it. In the newly fused part of the antimony at the base of the capillary a fine wire about 5 cm long is inserted. The final drawing out of the antinony microelectrode is carried out in an apparatus previously described [7]. It should first be drawn out at a distance of 10 mm from the site of contact of wire and antimony. The heating of the spirals and the small weight are determined empirically: as a rule, the heating must be somewhat stronger and the weight somewhat greater than the corresponding values used in preparing capillary microelectrodes. During the drawing out process care should be taken to ensure that there are no breaks in the antimony column at any point higher than the site of heating. Soda-glass was selected by us to lessen the probability of such breaks. The softening temperature of this type of glass is close to the melting point of antimony. The microelectrode, as a rule, does not solder. In the great majority of cases the antimony is broken off, after being extruded, flush with the glass. Thus, an antimony column is obtained insulated on all sides with the exception of the working surface. The electrodes are stored in air. They should be protected against dust.

* Biofizika 5: No. 3, 373–375, 1960.

In use, the microelectrode is attached by means of a rubber ring (11) to the adaptor (see Fig. 1 A). The lower part of this device is inserted into the copper tube 5, the split sleeve 3, slides over a segment of this copper tube about 15 mm long. At right angles to the sleeve a tube is soldered, and has a pinch-cock into which the arm of the micromanipulator is inserted. By means of the latter the whole system can be moved in all directions. The other end of the shielded cable is connected to the input of a Dubridge-Bardt type electrometer which has been previously described [8]. The resistance of the input of the electrometer was brought up to $10^{12}$ Ω. The instrument was supplied from lead-acid accumulators with a capacity of 270 A/hr. The drift of the instrument did not exceed 60 μV per hr.

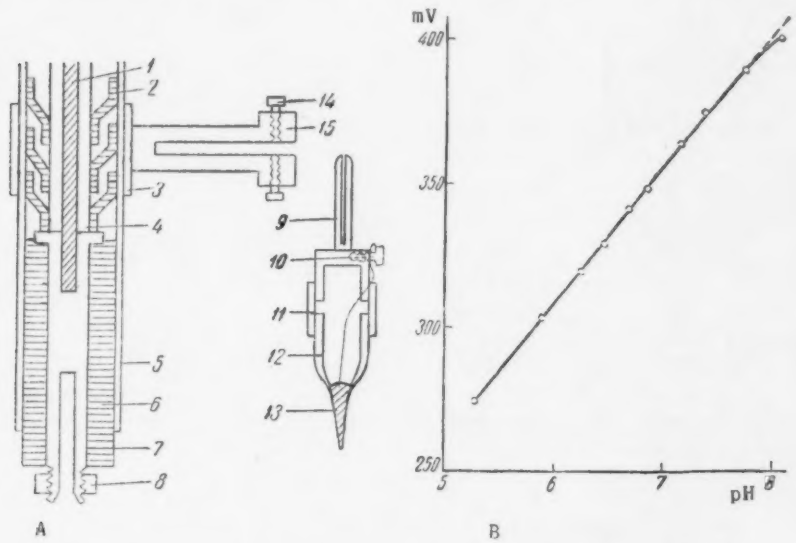


FIG. 1. (A) Diagram of adaptor and microelectrode. (1) lead; (2) porcelain ferrule; (3) slit sleeve; (4) lead insulation; (5) copper tube — shield; (6) porcelain tube — insulation; (7) clamp; (8) nut; (9) pin; (10) contact for clamp; (11) rubber ring; (12) microelectrode; (13) antimony; (14) screw; (15) tube for securing adaptor on arm of micromanipulator. (B) Potential of antimony microelectrode as a function of pH of buffer solution.

Investigation of the properties of the antimony microelectrode was made in Sörensen's phosphate buffer [9] within the pH range 5·28–8·04. With the electrometer input short-circuited, the microelectrode together and the calomel half-cell was alternately immersed into small vessels containing buffer solutions of different pH values, the short-circuit is removed and the potential of the antimony microelectrode relative to the calomel electrode is compensated. After each measurement both electrodes are washed in distilled water and by the buffer solution in which they are next to be dipped.

A graph relating the potential of the antimony microelectrode to the pH of the medium is given in Fig. 1 B. It has a negative potential in relation to the calomel half-cell. The graph was compiled on the basis of investigations of the properties of 30 microelectrodes with diameters of from 1 to 20 μ. The greatest mean square deviation of 3·3 mV from the curve gives an accuracy for method of 0·07 of a pH unit. Within pH range from 5·28 to 7·73, 50 mV is equivalent to one unit of pH. A certain sag of the curve in the alkaline region is characteristic of antimony electrodes and is apparently explained by the partial solution of the antimony oxide.

Of special importance in work with antimony microelectrodes is the value of the input resistance of the electrometer. During the investigation, it was seen that the current density passing through

the antimony electrode must not exceed definite values. With a higher current, the resistance of the electrode sharply rises and becomes commensurate with the load resistance. From experience the load resistance, i.e. the input resistance of the electrometer, must exceed the resistance of the microelectrode by about two orders of magnitude. With a diameter of microelectrode exceeding 5 μ, stable indications may be obtained with an input resistance of $10^{10}$ Ω. However, even for the microelectrode of 5 μ the indications become unstable in alkaline medium. The resistance of such electrodes reaches values of $10^8$–$10^9$ Ω. Since for a pH of 5·28–8·04 the e.m.f. changes within limits of 275–410 mV, the current density for such electrodes may reach 200 μA/cm², which already exceeds the permissible values. Electrodes with a diameter of about 1 μ give stable indications only if the input resistance of the electrometer has a value of the order of $10^{12}$ Ω. The current density in this case for the above pH range does not exceed 50 μA/cm².

The antimony microelectrode proposed is relatively simple to prepare, handle and store and gives stable indications provided a recording system is used with sufficiently high input resistance. The high resistance of the electrodes themselves is a certain drawback in the proposed method of determining the intracellular pH since it requires the use of an instrument with a long time constant.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# METHOD OF EMBEDDING HISTOLOGICAL MATERIAL IN METHACRYLATE*

L. S. GOLD'IN and V. S. PETROV

Bekhterev Psychoneurological Institute, Leningrad

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THE use of methacrylate for embedding histological material was proposed by Newman, Borysko and Swerdlow [1], and is one of the most outstanding events in the field of electron microscopy in the last 10 years.

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However, from the very outset it was known that embedding in methacrylate is often unsuccessful and the preparations obtained by such defective embedding are unsuitable for study.

Borysko [2] who has done extensive research on this problem, concluded that the greatest damage to cells occurs at the end of polymerization and depends on the unequal degree of compression of the polymer formed within the cells and outside them. In order to eliminate this phenomenon, Borysko proposed that embedding be done in partially polymerized methacrylate at a temperature of 60–80°. He assumed that the polymerizing monomer remains for a longer time in the liquid state and the tensile stresses developing between the cells and the intercellular spaces are removed by the flow of methacrylate from regions under higher to regions under lower pressure.

Moore and Grimley [3] noted that uniform compression of the monomer on conversion to the polymer may cause only a general compression of the whole preparation, without producing considerable damage in it. Damage arises as a result of the unequal polymerization of the methacrylate in the tissues and the inhomogeneity of the material thereby obtained. Since polymerization depends on the action of a small number of active molecules over a comparatively lengthy interval of time, it is extremely sensitive to the traces of substances acting as initiators or inhibitors.

VOL. 5 1960

Agents changing the concentration of active centres by a value of $10^{-12}$ mole may change the course of the reaction. Therefore, it is extremely important to maintain controllable standard conditions in biological embedding. In methacrylate treated in the usual manner oxygen and water remain in the dissolved state. Of these substances the most harmful is oxygen, which, depending on its amount in the monomer, can form the peroxide or oxide. In the first case oxygen acts as an inhibitor retarding the growth of chain molecules. In the second case, the oxygen accelerates growth of molecular chains. The authors removed the water by filtering the methacrylate through anhydrous sodium sulphate; the oxygen was removed by evacuating the dehydrated methacrylate at a pressure of 10 mm Hg. The methacrylate freed oxygen is stored in a dark glass vial at a temperature of +5° in an atmosphere of nitrogen. The covers of the gelatine capsules used for embedding are punctured with a needle and the capsules themselves, with the material, are placed in glass tubes which are filled with nitrogen and stoppered.

Moore and Grimley carried out polymerization at 43° for 18 hr.

Bachmeyer and Schreil [4] embedded the material in a few drops of methacrylate. To expel the dissolved ovygen they filled the gelatin capsules with carbon dioxide.

Bearing in mind the factors noted by these workers we decided to see how the quality of embedding is affected by preliminary heating of butyl methacrylate to the boiling point. This approach was based on the premise that butyl methacrylate boils at a temperature of 160° which is considerably higher than the boiling point of water. The small volume of water which remains in the methacrylate even when most carefully dehydrated must, on heating to 160° escape. At the same time, on boiling methacrylate all the gases dissolved in it must be expelled including the oxygen. With brief boiling, without initiator, methacrylate is partially polymerized and after cooling takes on a syrup-like consistency.

We carried out the embedding process as follows:

(1) Preliminary treatment of the butyl methacrylate or a mixture of it with methyl methacrylate, removal of inhibitor, washing and dehydration were carried out according to the method of Newman, Borysko and Swerdlow [1].

(2) The necessary amount of methacrylate was measured and transferred to a flask. The fllask must be of such a size that the hight of the liquid methacrylate in it is not greater than 2–3 cm. The latter was placed on an electric plate, or some other heater, heated to boiling and immediately removed from the heater at the moment of boiling. With longer boiling methacrylate sets firmly after cooling and becomes unsuitable for embedding. The methacrylate was cooled to room temperature whereupon it assumed the consistency of a syrup. It was slightly thicker than an 8% solution of celloidin. When a glass rod is dipped into it the methacrylate must fall in drops.

(3) The requisite amount of benzoyl peroxide (1–2 per cent) was weighed out, added to the methacrylate cooled to room temperature and left for 1–2 hr. Then, the benzoyl peroxide was carefully ground and crushed with a glass rod. This operation should be performed without abrupt movements to avoid

forcing air into the methacrylate. After the benzoyl peroxide is well ground, up, the vial with the methacrylate is carefully sealed and left until solution is completely effected, usually until the next-day since the benzoyl peroxide dissolves slowly in partially polymerized methacrylate.

(4) The material for embedding was treated by the usual procedure for electron microscopy; fixation in osmium buffer solution according to Palade [5] or by the modification of Caulfield with addition of sugar [6] or by another technique, then dehydrated in alcohols of increasing strength.

After dehydration the preparations were transferred for 30 min–1 hr to a mixture of equal parts of absolute alcohol and non-prepolymerized butyl methacrylate, then to two changes of pure non-repolymerized butyl methacrylate, to remove the alcohol. The preparation was left in each of these changes for 30 min–1 hr.

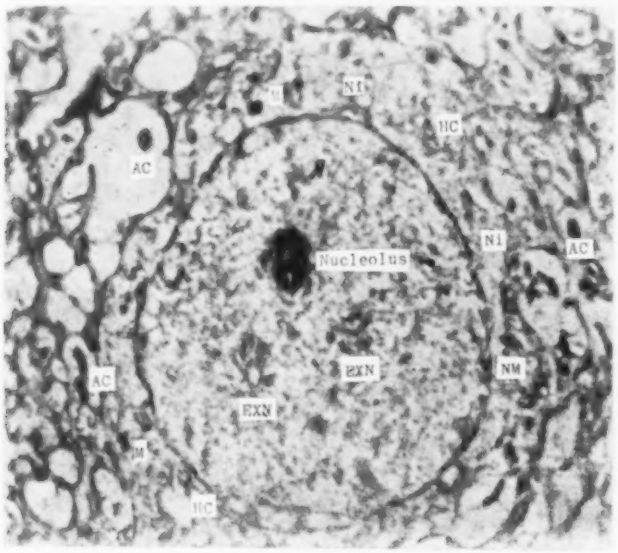


FIG. 1. Nerve cell from fifth layer of cortex of motor analyser of large cerebral hemispheres of white rat. (N) nucleus; (NK) nucleolus; (EXN) extra nucleolus; (NM) membrane of nucleus; (HC) Holmgren's canals; (AC) axial cylinders of nerve fibres; (Ni) Nissl bodies; (M) mitochondria. Magnification ×12,000; EMZ microscope.

(5) The preparations were transferred to the gelatine capsules filled with prepolymerized butyl methacrylate. Since the preparations usually do not automatically fall to the bottom of the capsule, they should be carefully lowered to the bottom with a needle, the tip of fine forceps or a very fine glass rod. Care should be taken to avoid forcing air bubbles into the methacrylate. The gelatine capsules are then covered and the preparations left at room temperature until the next day so that the prepolymerized methacrylate is able to impregnate the preparation completely. The preparations are placed in an incubator for 24–36 hr. We carried out polymerization at the temperature of 55–60°, as recommended by Borysko.

The results were considerably better than those given by ordinary embedding in methacrylate. Polymerization proceeds more uniformly with no bubbles in the capsules. On simultaneous polymerization of 100 and more preparations, scarcely any capsules were found with poor polymerization or insufficient density of polymer. The preparations cut better with a glass knife than do preparations embedded in the usual way.

As compared with the usual method of embedding, a much larger yield of sections suitable for our use was obtained; sections of the brain tissue and other parts of the nervous system studied by us, were in a satisfactory and good condition. An idea of the results which can be obtained is given by the microphotographs in Fig. 1 and 2. The material was fixed by the Palade method.

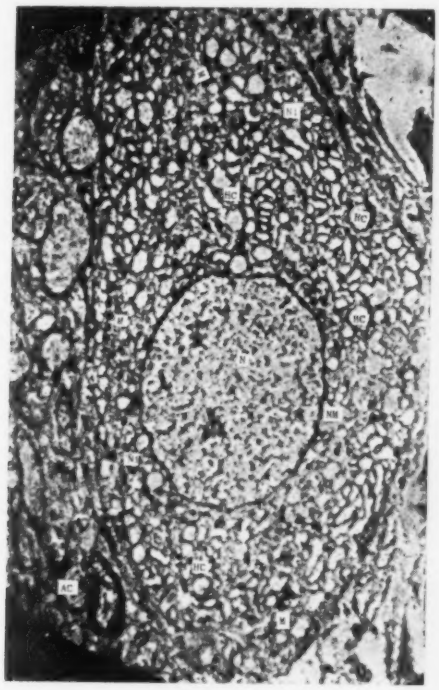

FIG 2. Sympathetic nerve cell of celiac ganglion of white rat. Magnification ×6000. EMZ microscope. Symbols as in Fig. 1.

The first photograph at a magnification of ×12,000 shows a nerve cell of the fifth layer of the cortex of the motor analyser of the white rat. The nucleus of the cell (N) is round in shape. The nuclear contents are in a finely dispersed state and evenly distributed throughout the nucleus. The nucleolus is small in size and oval. In addition, in the nucleus two other small conglomerations of granular elements (EXN), apparently extra nucleoli in the formative stage, are visible. The membrane of the nucleus is thin, not exceeding 0·03 μ in thickness. The cell protoplasm consists chiefly of very small granules which in many places form conglomerates probably constituting Nissl bodies (Ni). In it are also visible larger granules of an oval and round shape constituting mitochondria (M). Around the nerve cell are numerous nerve fibres, sectioned principally in a transverse or diagonal direction, and having an oval, elongated or round shape. Within most of these formations, dark oval or round bodies (DB) are visible and these are the axial cylinders of nerve fibres cut in the transverse or diagonal direction. The nerve cell is in the most intimate morphological relation to the nerve fibres. The morphological picture brought out in this photograph is in accord with that known from the morphology revealed by light optics.

Figure 2 depicts a sympathetic nerve cell from the coeliac ganglion of the white rat at a magnification of ×6000. The cell is elongated in shape, which is often observed in cells of this ganglion by light optics. Corresponding to this, the nucleus of the cell (N) is also oval. The internal structure of

the nucleus is similar to that in the preceding cell. There is no nucleolus; apparently, it did not come within the section. The membrane of the nucleus is visible over its entire course. In it, in some places there are impressions which apparently were formed at the time of cutting. The protoplasm of the cell in question differs considerably in structure from that of the preceding cell. All of it is pierced by slits of a round, oval or elongated shape. Around each of these slits there is clearly visible the membrane separating each of these formations from the adjacent allied formation, and from the ground substance of the protoplasm located around many of these slits. The same slits can also be seen in the protoplasm of the cell depicted in the preceding Figure. However, there they are rarer and their size is considerably less than for the cell in Fig. 2.

We assume that these formations correspond morphologically to the Holmgren's canals described at the end of the last century, the existence of which has been questioned by many workers. Sometimes they have been identified with the reticular apparatus of Golgi. Our findings show that these canals do in fact exist and must be distinguished from the reticular apparatus. They are more strongly developed in the protoplasm of certain types of nerve cells.

Around the nerve cell described, there are many nerve fibres sectioned in the most varied directions. The photographs under consideration do not display structural damage or distortions attributable to forces developing during polymerization of methacrylate. This can be judged from the shape of the nucleus of the nerve cells in usual histological preparations and also from the shape of the Holmgren's canals and the transversely sectioned nerve fibres.

VOL
5
196

## SUMMARY

(1) The quality of embedding in methacrylate is improved when it is subjected to preliminary prepolymerization and removal of oxygen and water.

(2) Brief heating of methacrylate to boiling point is an effective means of eliminating damage to tissue elements caused during polymerization, gas bubble formation in the polymerizing block is avoided and uniform polymerization throughout the preparation is ensured. This gives more advantageous conditions for obtaining hyper-fine sections good quality.

*Translated by* A. CROZY

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# LETTERS TO THE EDITOR

## RELATION BETWEEN THE ELASTIC PROPERTIES OF NERVE FIBRE AND CERTAIN FUNCTIONAL CHARACTERISTICS*

K. M. L'VOV, L. P. KAIUSHIN and R. G. LIUDKOVSKAIA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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OL.
5
960

AN ambiguous relation between the electrophysiological characteristics of an excitation wave in the nerve and temperature was noted long ago [1]. This is expressed in the fact that at a given temperature (for the same nerve) there can exist several different values of amplitude of the action potential, velocity of conduction, etc. This phenomenon is commonly associated with deterioration in the functional state of the tissue [2]. Therefore, from a large number of experiments some are selected in which such a type of relation is well-defined or, the investigation is carried out with a single change in temperature.

In our view, an analysis of this relationship requires a different approach. The nerve fibre is made up of polymer substances. The special elastic and plastic properties of the nerve fibre must inflence the character of the chemical and physiological reactions in the nerve and its mechanical properties The connexion between excitation of the nerve and certain mechanical changes has been studied in a number of papers [3–8]. We assumed that the usual electrophysiological characteristics of the spreading of excitation on change in temperature must form hysteresis loops, display relaxation, etc., analogous to the mechanical parameters of high molecular substances. Experiments have been carried out on the isolated giant axon of the cuttlefish to study the relation between the velocity of conduction of excitation and temperature.

The temperature of the nerve was varied by passing heated or cooled water through a water jacket surrounding the chamber containing the nerve. The temperature was measured with thermocouples in the immediate vicinity of the axon.

In different experiments the direction of the temperature course was varied from 1 to 5 times over a definite range. The minimum temperature to which we could take the nerve without it losing excitability in some experiments was 8°, the maximum 44°. The rate of change in temperature in all experiments remained virtually constant at 1·5° per min.

The velocity of the excitation wave was determined as follows. The axon, dissected out with a small number of surrounding fine fibres, was placed in a humid chamber on platinum electrodes. The distances between the stimulating electrodes and between the internal stimulating electrode and the recording ones were each 10 mm, and the distance between the recording electrodes about 3 mm. By varying the polarity of the stimulating pulse, we excited the nerve sometimes over one and sometimes over the other electrode. From the results of the experiment we determined the times the excitation wave covered a distance of 10 mm and calculated its velocity.

In all experiments, qualitatively reproducible results were obtained. The typical relation between the velocity of the excitation wave and temperature is depicted in Fig. 1 and 2. It is clear that there

* Biofizika **5**: No. 3, 379–381, 1960.

is no well-defined relation between the speed of conduction of the excitation wave and the temperature and that the curves are of a hysteresis character. For example, in Fig. 2 for a temperature of 20° there are two values of the velocity of the spread of excitation (depending on the direction of change in temperature).

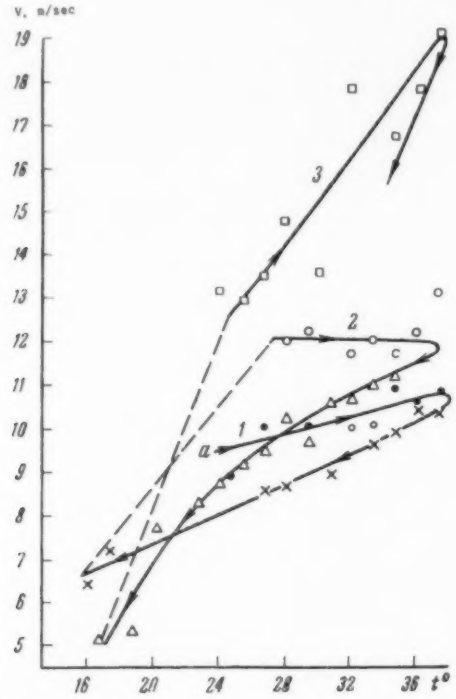


FIG. 1. Relation between velocity of spread of wave of excitation and temperature. 1, 2, 3, cycles of change in temperature.

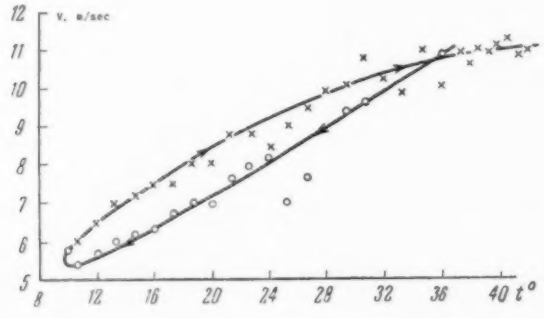


FIG. 2. Variation of velocity of spread of wave of excitation with temperature.

Fig. 3 gives the results of an experiment in which the temperature after being changed from 26° to 35° was kept constant for 4 min. It is clear that despite the constancy of temperature the velocity greatly increased.

In the same experimental conditions the length of the excitation wave (by which we mean the length of the electrically active portion of the wave) at a fixed temperature also increased so that the time taken by the excitation wave to pass the recording electrodes (duration of excitation wave) changed little (Fig. 4).

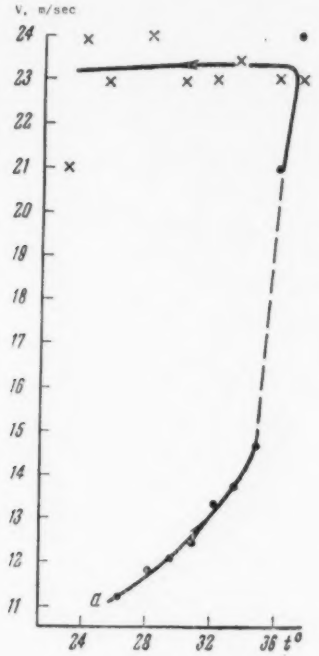


FIG. 3. Relaxation of velocity of spread of excitation wave on fixing temperature after changing it. At 35° temperature not changed for 4 min.

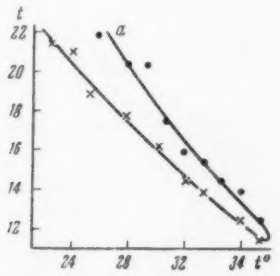


FIG. 4. Duration of excitation wave as a function of temperature.

We associate the relation between the velocity of conduction, length of excitation wave and temperature with the relaxation of the structural stresses developing in the nerve. The constancy of the duration of the excitation wave indicates the absence of relaxation changes for this parameter at constent temperature. The relative divergence of the branches of the curve (Fig. 4) is inconsiderable compared with the curve in Fig. 3.

In some experiments, on reaching the lowest or highest possible temperature the excitability of the nerve was lost, but it was restored by the corresponding increase (Fig. 1) or decrease in temperature in the case of heating from low temperature the velocity of propagation of the wave appreciably rose and the threshold of excitability dropped.

Thus, for successive temperature cycles, the functional properties of the nerve are improved. As a result, the maximum values of velocity reached 20–25 m/sec as against the initial velocities (temperature 25°) of 10–12 m/sec. Consequently, we cannot attribute the observed hysteresis and relaxation features of the parameters of the nerve to a deterioration in function.

Our findings confirm the important rôle of the high polymer component of the nerve substrate in the formation and conduction of the wave of excitation along the nerve. Hysteresis and relaxation characteristics are common to polymer systems and in our view it is precisely these features which impose a deep imprint on the relation between the velocity, length of the excitation wave and the temperature. Similar findings have been obtained in studies on the excitability threshold, spike amplitude and velocity of conduction for the nerves of warm-blooded animals and frogs [9–11]. However, the authors do not give any explanation of their findings.

The experiments were carried out in the town of Tsing-dow, at the Institute of Oceanology of the Chinese People's Republic Academy of Sciences.

We wish to express our gratitude to Prof. Fen De-pei and Tun Tsi-Tsao and also their colleagues for their help in the work and providing facilities for the experiments.

*Translated by* A. CROZY

VOL
5
196

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* There is a translation into English of this work.

# CURRENT EVENTS

## INTERNATIONAL SYMPOSIUM ON ELECTRO-RETINOGRAPHY HELD IN CZECHOSLOVAKIA*

IN recent years a series of international conferences has been held on the problems of electroretinography (Hamburg, 1956; Erevan, 1957; Bethseda 1958). The last symposium was held in the picturesque resort of Czechoslovakia — Luhacovice — from 24 to 26 September 1959. It was organized by the

* Biofizika **5:** No. 3, 382–384, 1960.

Opthalmological Section of the Czechoslovak Purkinje Medical Society. The President of the Organizing Committee of the Symposium was the outstanding specialist in the field of electroretinography, Prof. J. Vanicek, who together with other Czechoslovak friends did everything possible to ensure its success.

The symposium on electroretinography in Luhacovice was on a high scientific level. Many prominent workers in the field of experimental and clinical electroretinography came to Czechoslovakia and submitted reports. There were scientists (opthalmologists, physiologists, biophysicists) from the U.S.S.R., U.S.A., Czechoslovakia, Belgium, France, Italy, the German Federal Republic, the German Democratic Republic, Switzerland, Bulgaria and other countries.

The symposium was formally opened by the inaugural address of Acad. J. Kurts — President of the Czechoslovakian Purkinje Society of Opthalmologists. Then, speeches of welcome to the members of the symposium were made by the Vice-President of the International Society of Clinical Electroretinography, Prof. G. Francois (Belgium), the General Secretary of the same society, Dr. Henkes (Holland), Prof. B. N. Melik-Mus'ian (U.S.S.R.) and President of the Organizing Committee, Prof. J. Vanicek (Czechoslovakia).

OL.
5
960

The first two sessions of the symposium were devoted to problems of experimental electroretinography with Prof. C. Francois (Belgium) and G. G. Demirchoglian (U.S.S.R.) in the chair.

At the symposium a report "On the mechanisms of origin of the e.r.g." was delivered by Demirchoglian. The report gave a critical review of the most widely held theory in the West of the origin and rôle of the electrical phenomena of the retina evolved by the prominent Swedish physiologist R. Granit. This theory based on recognition of the existence of several hypothetical e.r.g. components is extremely schematic, and divorced from the modern advances in biophysics and biochemistry of the retina. In opposition to this the speaker described an attempt at substantiating the metabolic photochemical theory of the origin of the e.r.g. It should be noted that in a number of other reports and also in the discussion Granit's theory came in for sharp criticism (Prof. Muller-Limroth, etc).

The paper of Prof. Jacobson (New York, U.S.A.) was devoted to standardization of methods of clinical electroretinography. For this purpose he recommended use of the cathode oscillograph for recording the e.r.g. in response to light stimuli of variable chromacity. The author also uses a special safety device in the circuit of the amplifying unit, since in one case of disturbance in the circuit the electrical current penetrated the patient's eye causing damage to the corneal epithelium.

Gutt (Munster, German Federal Republic) carried out an investigation of the intraretinal potentials and revealed the existence of oscillations (10–20 oscil./sec) against the background of the "*b*" wave of the e.r.g.

The symposium heard with great interest the report by Prof. Muller-Limroth (Munster, German Federal Republic). The report analysed recent investigations in the field of electroretinography with a very detailed account of the results of intraretinal investigations with microelectrodes. Drawing a parallel between photochemical phenomena in the retina and the processes of photosynthesis, Muller-Limroth reached the conclusion that the primary photochemical process indisputably generates the receptor potential underlying the e.r.g. It is of the simplest form and may be both positive and negative in relation to the type of cones. It is supplemented by the potentials of the bipolar cells and other elements of the complex retina of vertebrates. In the view of the speaker, major importance must be attached to the pigment epithelial layer in which complex metabolic processes occur. Therefore, any disturbance in metabolism leads, in the first place, to disturbances in the pigment epithelium. Here the so-called "*c*" wave of the e.r.g. probably arises.

Best (Bonn, German Federal Republic) reported on his study of the "off" effect. He stated that he had been able to observe a stronger "off" effect in the e.r.g. on stimulation with red than with blue light.

Ass. Prof. Straub (Hamburg, German Federal Republic) reported on his experiments on the effect of cigarette smoking, coffee drinking and inhalation of "laughing gas" on the magnitude of the e.r.g. of the human eye. It was established from these experiments that smoking causes intensification of the e.r.g., coffee also led in the majority of cases to intensification of the e.r.g. and inhalation of a mixture

of 60 per cent "laughing gas" and 40 per cent oxygen gave indeterminate results. The speaker assumed that the so-called "spontaneous" oscillations of the e.r.g. observed in clinical electroretinography may be explained by such factors.

Van-der Twil (Amsterdam, Holland) studied the electrical response of the retina to modulated light. The experiments were conducted on cats and man with use of a contact lens. The frequency characteristics of the electrical reactions of the retina were recorded and the non-linear character of the retinal processes determined.

Prof. Voneshova (Zagreb, Yugoslavia) presented an original paper on the application of certain principles of quantum mechanics to study the processes occurring in the photoreceptors and those associated with the formation of the e.r.g. An interesting attempt to give a mathematical interpretation to the e.r.g. was also made by Dr. Kozucek (Brno, Czechoslovakia).

Prof. Virt (Rome, Italy) read a paper on the slow components of the e.r.g.

In the report of Cand. Biological Sciences M. A. Allakhverdian (Erevan, U.S.S.R.) new material was presented on conditioned reflex changes in the e.r.g. The speaker worked with rabbits and obtained conditioned reflex changes in the height of the "*b*" wave of the e.r.g. after 50–100 combinations of light with a bell. The presence of efferent influences of the large (cerebral) hemispheres on the retina of the eye was thus established.

Samson-Dolfuss (Rouen, France) recorded the e.r.g. and e.e.g. of man using various conditions of illumination by means of a stroboscope. The absence of an interrelation between the shape and size of the retinal potentials and the response potentials of the brain was established.

Asher (Birmingham, England) presented a paper on the relations between the "*a*" and "*b*" waves of the e.r.g. and subjective sensation of the intensity of light. It was found that at low light intensities the amplitude of the e.r.g. "*b*" wave was directly proportional to the logarithm of the light intensity. However, at high intensities it remained at a constant level which did not vary with further increase in the intensity of the flash. As opposed to this, the "*a*" wave of the e.r.g. was absent at low intensities increased up to maximal flash intensity. Performing experiments on himself, he noted that the subjective sensation of light intensity constantly increased right up to the highest attainable intensities, which demonstrates a difference in their behaviour. The speaker holds to the view that the e.r.g. is produced by scattered light in the retina.

Berges Munster, German Federal Republic) made an electroretinographic study of the mechanism of action of carotenoids on the retina. He found that on administration of vitamin A or helenin to frogs the "*a*" and "*b*" waves of the e.r.g. diminished. He also made a flash photometric determinations of the ionic shifts (Na — K content) occurring at the same time in the retina but found no correlation between ionic changes and shifts in the e.r.g.

Profs. Vavra, Sverak and Peregrin (Gradets-Kralovy, Czechoslovakia) gave a report on the technique of bilateral human electroretinography. This method permits observation on the oscillograph screen of the vectoretinogram and determination of the direction of the retinal axis in normal and pathological states. The new technique promises to render useful service in the diagnosis of diseases of the retina.

The second communication by Peregrin and Sverak was devoted to the application of a method of stimulation of the eyes with flickering light (20 sec at a frequency of 15–20 cycles/sec) in order to activate the retina. They found an increase in the amplitude of the e.r.g. "*b*" wave after such "activation". Moreover, it was not possible to discover eyes with a pathological change in this property. It is proposed to use this new functional test in electroretinography.

The paper by Ts. M. Avakian (Erevan, U.S.S.R.) was concerned with the effect of X-rays on the functional state of the retina.

The two other sessions of the symposium held under the chairmanship of Prof. B. N. Melik-Mus'ian (U.S.S.R.) and Prof. Vavra (Czechoslovakia) were devoted to the problems of clinical electroretinography. Here, the main report was delivered by Prof. Vanicek (Brno) who summed up the results of the clinical use of e.r.g. methods. He pointed out that at present the e.r.g. facilitates the work of clinicians with regard to indications for surgery in turbidities of the cornea and crystalline lens. The

e.r.g. also enables one to distinguish congenital from functional nyctalopia. The e.r.g. is also necessary for diagnosis of tapetoretinal diseases. The prognostic value of this method is high also in retinal detachment. The e.r.g. method is an aid in blood circulation disorders of the retina. The speaker also emphasized that the e.r.g. has not yet gained an important place in the diagnosis of such diseases as glaucoma, colour blindness and retroretinal diseases.

The report of Prof. B. N. Melik-Mus'ian (Erevan, U.S.S.R.) described the experience gained in the application of e.r.g. in glaucoma. The method has helped to determine the presence or absence of colour sensitivity of the retina in cases of absolute glaucoma. In the initial stages of glaucoma, an e.r.g. in response to red light is absent. It is intensified after use of anti-glaucomatous substances. The speaker considers that there is need for further systematic research to test the value of the e.r.g. in glaucoma. The application of e.r.g. in glaucoma was also dealt with in the report of Aladzhev (Sofia, Bulgaria) who in each patient studies the scotopic, photopic and flickering e.r.g. together with a sample of water. From an analysis of this material the speaker concluded that early stages of glaucoma cannot be diagnozed by electroretinography.

VOL. 5 1960

Gayle and Bouet (Marseilles, France) presented data on dynamic electroretinography in myopia. In their work they used a number of functional tests (dark adaptation, red light, weak and strong illumination, flickering illumination and determination of the retinocortical time). In these investigations with a weak degree of myopia the e.r.g. is normal, with a strong degree low. It was also established that in myopic retinopathy the photopic system of the retina is affected earlier than the scotopic one. Study of the e.r.g. in myopias was also described in the paper by Profs. Franchechetti and Diterle (Geneva, Switzerland). They carried out statistical treatment of the material and discovered a linear relation between the degree of myopia and the reduction in the e.r.g. "*b*" wave.

Prof. Schmeger (Erfuhrt, German Democratic Republic) reported on the application of the e.r.g. "*a*" wave in the eye clinic. He used a very bright electronic flash as light stimulus. Specific changes were found (decrease) in this wave only with retinal detachment. This result confirms the findings of many workers on the resistance of the "*a*" wave to various influences.

The report of Prof. Francois and de Rouque (Ghent, Belgium) was devoted to disturbances in the e.r.g. in daltonism.

Papst (Hamburg, German Federal Republic) reported on the use of flickering electroretinography in ophthalmic practice. He considers that the determination of the amplitude of the e.r.g. "*b*" wave at low frequencies of light flickers (2–3 oscil./sec) is more accurate and useful than e.r.g. recorded in response to separate stimulations. Thus, for example, with blood circulation disorders in the retina the e.r.g. at flicker frequencies of 2–3 cycles/sec was reduced whereas the usual e.r.g. remained normal.

Henkes (Rotterdam, Holland) reported on a new method of recording the e.r.g. from small areas of the retina of the human eye. Using a frequency analyser, he established the presence of electrical responses in cases of primary pigment degeneration of the retina, although in response to even a very bright stimulus the e.r.g. was absent. In order to exclude scattered light in clinical investigations the author used very weak light. The signal/noise ratio was improved by use of a new type of suction contact lens, a special attachment for oscillograph screen photography and integration of the e.r.g. responses.

At the end of the symposium the members visited the towns of Brno, Prague and Karlovy Vary where they became acquainted with the eye clinice and research institutes of Czechoslovakia.

The International Symposium in Luhacovice reflected the wide development of the electrophysiological methods of research into the complex functions of the retina and the successful introduction of electroretinography into ophthalmology.

G. G. DEMIRCHOGLIAN
*Translated by* A. CROZY

VOL
5
196

# LUMINESCENCE SPECTRA OF AROMATIC AMINO ACIDS AND PROTEINS*

Iu. A. Vladimirov and E. A. Burshtein

Faculty of Fundamental Biology, Lomonosov State University, Moscow

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OL.
5
960

Study of the luminescence of biologically important compounds is of interest from several points of view. Firstly, the luminescence spectra enable one to detect a given luminescent compound and directly estimate its state in undamaged tissue. Secondly, the position of the maxima in the luminescence spectra corresponds to the levels of electron excitation at which the molecule can maintain and accumulate absorbed energy. Finally, spectral investigations of the luminescence of complex systems when combined with other methods make it possible to trace the fate of the energy quantum (in the simplest case light quantum) in a complex system: the migration of energy between different molecules or groups of the same molecule, storage of energy at metastable levels, quenching of the quantum, its expenditure on chemical reactions, etc. Therefore, it is natural that detection of luminescence in aromatic amino acids [1–3] and proteins [2, 4] engaged the attention of a number of investigators.

Theale and Weber [5] measured the fluorescence spectra and fluorescence-excitation spectra of aromatic amino acids in solution. Barskii and Brumberg [6] and Vladimirov [7] investigated the luminescence of crystals of aromatic amino acids. Vladimirov [7], Vladimirov and Litvin [8], Steele and Szent-Gyorgi [9] measured the fluorescence and phosphorescence spectra of proteins. The fluorescence excitation spectra of proteins has been studied by Konev [10–12], the excitation spectra of crystals of aromatic amino acids by Vladimirov [13, 14] and the phosphorescence excitation spectra of proteins and amino acids by Vladimirov and Litvin [8].

Of great importance in the study of the nature of protein luminescence is accurate measurement of the spectrum, this is particularly the case for defining the link between the luminescence of the protein and the physico-chemical state of the macromolecule. However, in many of the investigations mentioned, especially the protein luminescence spectra [7, 9, 15, 16], it is difficult to estimate the structure of the spectra and the precise position of the maxima from the published data. In addition, to our knowledge, no systematic comparative investigations have been made of the luminescence spectra of proteins and aromatic amino acids in identical conditions at room and low temperatures and over a wide spectral region which is fundamental for elucidating the

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problems of the energy interaction of the different groups of the protein molecule and for elucidating the relation between the luminescence spectra and the state of the protein macromolecule. The object of the present work was an attempt to bridge this gap.

## METHODS

Luminescence spectra were studied at room temperature and on cooling to —140 to —160° in the 280 to 520 mμ region with an instrument mounted at the base of an SF-4 monochromator (Fig. 1). The source of the exciting light was a 1000 W PRK-7 high pressure mercury-quartz lamp, the light of which was focused on the cuvette containing the solution by means of a quartz flask filled with distilled water. The

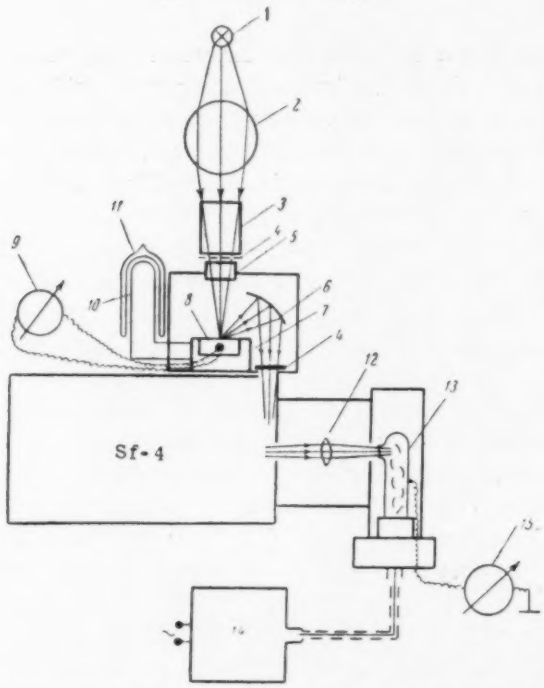


FIG. 1. Arrangement for measurement of luminescent spectra. (1) PRK-7 mercury lamp; (2) condenser; (3) gaseous filter; (4) BS-2 light filter; (5) liquid, light filter; (6) concave mirror; (7) cuvete holder; (8) cuvette containing solution; (9) galvanometer of thermocouple; (10), cold conductor; (11) Dewar vessel with liquid nitrogen; (12) quartz lens; (13) FEU-18 photomultiplier; (14) high voltage stabilizer and rectifier; (15) M-91/A galvanometer.

spectral region from 240–280 mμ was isolated for excitation by a 7 cm thick chlorine gas filter and a liquid Bechstrom filter (aqueous solution of cobalt and nickel sulphates) 2 cm thick. Thus, luminescence was excited in the main by the light of the 254, 265 and 275 mμ mercury lines and, partially by the 280 mμ line, while in the 282–520 mμ region mercury lines were virtually absent. The luminescence light was collected by

an aluminium concave mirror and focused on the inlet slit of the monochromator. A BS-2 light filter which absorbed the light of the region of excitation was placed in front of the entry to the monochromator. In order to allow for scattered light the BS-2 filter was placed along the path of the exciting light. When measuring of the fluorescence spectra of phenylalanine the BS-2 filter was not used, and the solvent served to check the scattered light. On passing from the monochromator, the luminescent light was additionally focused by a quartz lens on to the photocathode of an FEU-18 photomultiplier fixed at the site of the SF-4 photocell. The photomultiplier was supplied by a high voltage rectifier and a stabilizer of the "Orekh" type. Most of the measurements were made with a photomultiplier voltage of 1100 V, at this voltage the level of noise was not more than 2 per cent of the signal. The photo-current was measured by means of a M-91 A galvanometer with a sensitivity of $1{\cdot}0\times10^{-8}$ A per degree. The error of measurement was not more than 0·4 of a division of the galvanometer scale. The measurements were made with monochromator slit widths of 0·01–0·1 mm which gave good resolution of the structure of the spectra. The aluminium cuvette containing the solution was closed by a quartz cover and was placed in a brass holder with a cold conductor cooled by liquid nitrogen. The temperature of the cuvette, determined by a thermocouple during the measurement of one spectrum, varied by not more than 5–7°.

Irradiation of the object by exciting ultra-violet led to changes in the luminescence spectra which were inconsiderable during the measurement of one spectrum (2–5 min), but in some cases, led to the appearance of additional maxima of the fine structure of the spectra (the maxima, intensity of which increased with time of radiation, are indicated in Table 1 by asterisks).

All the spectra were corrected for spectral sensitivity of the instrument measured in the region 280–380 mμ according to the fluorescence of a solution of dimethylaminonaphtho-sulphate, the quantum yield of which was taken as constant on excitation in the whole of this region [5], and measured in the region 280–520 mμ against the standard ribbon lamp.

The luminescence spectra of solutions of the aromatic amino acids, tryptophan, tyrosine and phenylalanine and the proteins, human serum albumin, chick egg albumin, human serum gamma globulin, actomyosin and zein*. The zein was dissolved in 60% ethyl alcohol; the actomyosin in 0·6 M KCl solution while all the other solutions were prepared in phosphate buffer (0·1 M $Na_2HPO_4$). The optical density of the solutions was 0·2–0·4.

## RESULTS AND DISCUSSION

The fluorescence spectra of the solutions of phenylalanine, tyrosine and tryptophan at room temperature are depicted in Fig. 2. They approximate to the spectra obtained by Theale and Weber [5]. The difference in the position of the maximum

* The serum and egg albumins and the gamma globulin were kindly made available to us by the laboratory of A. G. Pasynskii, zein by O. P. Osipova; the amino acids used were commercial grades; the actomyosin was isolated from rat muscle by the usual technique [17].

of fluorescence of tryptophan (353 mμ in our measurements and 348 mμ obtained by Theale and Weber) is possibly due to the higher alkalinity of the medium in our experiments. A reduction in the pH of the medium rendered the maximum at 348 mμ (Fig. 2) more distinct.

Table 1. Position of luminescence maxima of solutions of proteins and aromatic amino acids: at room (R) and low (L) temperatures

| Sample | Fluorescence | | Afterglow |
|---|---|---|---|
| | Middle of band | Maxima of fine structure | |
| Phenylalanine | | | |
| R | 284 | **282**, 288, 297 | — |
| L | 297 | 280, 287, **296**, 302 | **425**, 447, 490 |
| Tyrosine | | | |
| R | 304 | **304**, 308 | — |
| L | 298 | 298 | 378*, 387 |
| Tryptophan pH 8·2 | | | |
| R | 352 | 347, 354, 359*, 364* | — |
| L | 328 | 320, 326, 330, 334, 339, 344* 352, 390 | 406, 431, 458 |
| Tryptophan pH 5·5 | | | |
| R | 351 | 347, **354**, 359* | — |
| Serum albumin | | | |
| R | 338 | 326, 330, 334, **339**, 344* | — |
| L | 316 | — | 412, 436, 465 |
| Egg albumin | | | |
| R | 334 | 330, **334**, 388, 344* | — |
| L | 322 | 320, 326, 334 | 411, 437, 460–465 |
| Gamma globulin | | | |
| R | 336 | — | — |
| Actomyosin | | | |
| R | 335 | 326, **334**, 338, 343* | — |
| Zein | | | |
| R | 304 | 304, 308 | — |
| L | 301 | 299, 304, 308 | **387**, 430–433 |

* Maxima, the relative intensity of which increased an irradiation with ultra-violet. Data on fine structure of fluorescence spectra are only preliminary.

On lowering the temperature the fluorescence bands of the amino acids shifted somewhat (Fig. 3). For example, in the case of tryptophan with a reduction of temperature to —130 and —155°, there was a successive shift in the fluorescence band to the shortwave region (the centre of the band shifted to 330 and 328 mμ respectively). This gave a fine band structure (Table 1) which apparently reflects the position of the vibratory sublevels of the excited state.

Together with these effects, in all three amino acids in the frozen solutions luminescence appeared in the visible and near ultra-violet region (Fig. 3), which in the case of tyrosine and tryptophan was close to that studied by Steele and Szent-Gyorgi [9]

and by Isenberg and Szent-Gyorgi [18] for phosphorescence and, with respect to the position of the maxima, coincided with the spectra measured by Vladimirov and Litvin [8].

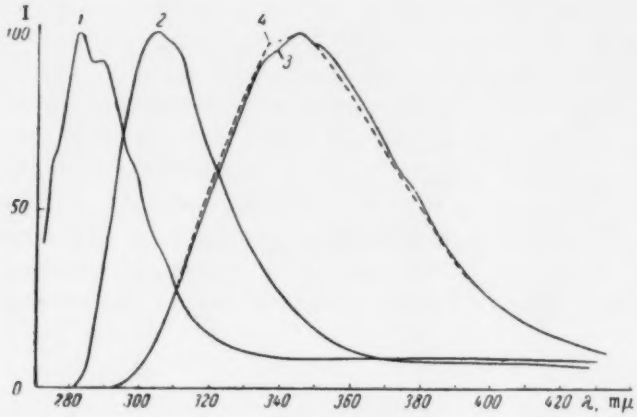


FIG. 2. Fluorescence spectra of aromatic amino acids at room temperature. (1) phenylalanine; (2) tyrosine; (3) tryptophan at pH 8·2; (4) tryptophan at pH 5·5.

The luminescence spectra of proteins at room and low temperatures are shown in Fig. 4 and 5. In the spectra of the proteins containing all three aromatic amino acids (serum and egg albumins, gamma globulin, actomyosin) both at room and at low temperature luminescent maxima corresponding to phenylalanine and tyrosine were absent, despite the high content of these in the proteins, e.g. human serum albumin contains 33 residues of phenylalanine, 18 of tyrosine and only 1 of tryptophan [19]. Luminescence of these proteins depended on the tryptophan, the emission spectra

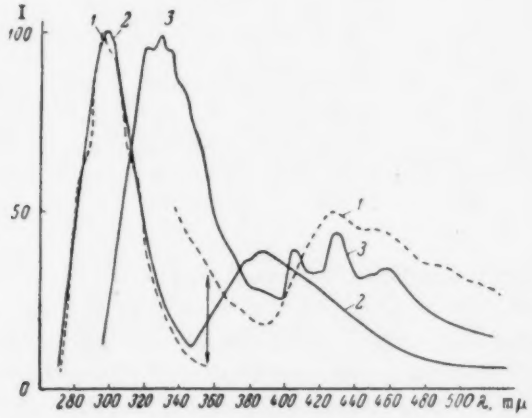


FIG. 3. Luminescence spectra of frozen solutions of aromatic amino acids. (1) phenylalanine; (2) tyrosine; (3) tryptophan. Longwave portion of curve 1 is given on larger scale.

of which in protein are somewhat shifted as compared with the spectra of solutions (Table 1). In the case of zein, containing only phenylalanine and tyrosine, the fluorescence spectrum was determined solely by tyrosine. In the low temperature afterglow spectrum of zein both tyrosine and phenylalanine components were detected.

The absence of phenylalanine and tyrosine maxima in the luminescence spectra of tryptophan-containing proteins has already been noted by Vladimirov [7] and also by Steele and Szent-Gyorgi [9] and has been attributed to migration of energy from phenylalanine and tyrosine to tryptophan. The close positioning of aromatic amino acids in the proteins (up to 100 Å) and the overlapping of the fluorescence spectra of phenylalanine and tyrosine with the absorption spectrum of tryptophan makes the migration of energy between the aromatic amino acids in protein highly probable [20–22], in similar manner to that observed in concentrated solutions and crystals of these compounds [13, 14]. On the other hand, such an assumption is inconsistent

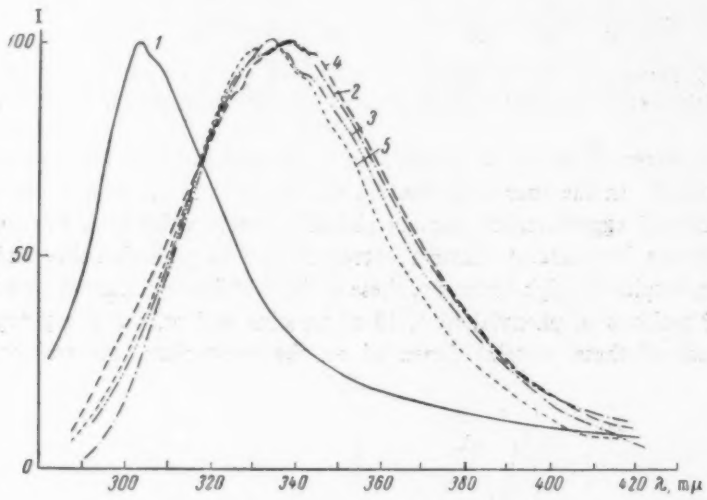


FIG. 4. Fluorescence spectra of proteins at room temperature. (1) zein (in 60% alcohol); (2) serum gamma globulin; (3) actomyosin (in 0·6 M KCl); (4) serum albumin; (5) egg albumin.

with the absence of phenylalanine and tyrosine maxima in the afterglow and fluorescence-excitation spectra of tryptophan-containing proteins [8] which indicates that the quantum absorbed by these amino acids in proteins is not emited in the form of luminescence of tryptophan but is dissipated into heat or expended on photochemical processes. Such an expenditure of the quantum absorbed by phenylalanine and tyrosine may be explained, it seems to us, not because of the incorporation of these amino acids into a polypeptide chain of the proteins [15] but by quenching of this luminescence by tryptophan, for example, during migration of energy. Support for this argument

is provided by the fact that in proteins not containing tryptophan: ribonuclease [15], insulin [9, 15] and zein, the tyrosine luminesces but in phycoerythrin and phycocyanin the light quantum absorbed by tyrosine is emitted in the form of fluorescence of the chromaphore group [23, 24].

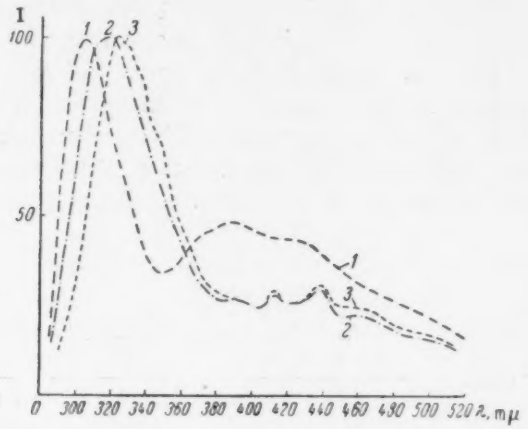


FIG. 5. Luminescence spectra of frozen protein solutions. (1) zein; (2) serum albumin; (3) egg albumin.

On inspecting the spectra of tryptophan-containing proteins a notable feature is the change in fluorescence of tryptophan when incorporated into the peptide chain. As Fig. 4 and 5 show, the fluorescence spectra of the proteins are shifted to the shortwave side, relatively to the spectrum of tryptophan solution, and in terms of position approximate to the spectrum of fluorescence of frozen tryptophan. The similarity is heightened by the fact that the position of the maxima is uniform in solutions of proteins and in the frozen solution of tryptophan (Table 1). Possibly, the restriction of the mobility of tryptophan when incorporated into the protein affects the spectrum in similar manner to the rise in the rigidity of structure on freezing. The fluorescence spectrum of the frozen protein solutions shifts still further to the shortwave side and is even closer to the spectrum of the frozen tryptophan solution (Fig. 5). The difference in the state of tryptophan in protein and in aqueous solution is also reflected in the shift of the afterglow spectra to the longwave side (Table 1).

However, the matter is not merely one of a difference in the luminescence spectra of protein and free amino acids. Comparison of the fluorescence spectra of different proteins shows some difference in the position of the bands which is possibly a reflexion of the different probability of emission from the individual vibratory sublevels of the excited molecule since the scarcely detectable fine structure of the spectra has uniform maxima for the different proteins. The fluorescence spectra of the various proteins also differed somewhat at a low temperature. These differences were minor but reproducible on repeat measurement. Possibly they are associated with the different character

of the bonds of tryptophan in the protein molecules or with different acid-base equilibrium in the direct environs of the tryptophan residue.

It was of interest to investigate the problem of the changes in the spectra of the tryptophan residue on denaturation of the protein molecule. For this purpose, measurements were made of the luminescence spectra of four proteins in the native and denaturated state (heating for 10 min on a boiling water bath). As Table 2 shows, the fluorescence spectra changed somewhat on denaturation, and in different ways for the various proteins. The changes in the system of hydrogen bonds, unfolding of the chains and changes in the configuration of the protein molecule as a whole, alter the local conditions around the tryptophan residues and are reflected in a changed luminescence spectra. The intensity of fluorescence on denaturation in the case of serum and egg albumins diminished and in the case of gamma globulin rose (Table 2).

TABLE 2. SPECTRAL CHANGES IN FLUORESCENCE OF PROTEINS ON DENATURATION

| Sample | Middle of fluorescence band, (mμ) | | Relative intensity of fluorescence, (%) | |
|---|---|---|---|---|
| | Native protein | Denaturated protein | Native protein | Denaturated protein |
| Serum albumin | 338 | 330 | 100 | 67 |
| Egg albumin | 334 | 336 | 100 | 69 |
| Gamma globulin | 336 | 339 | 100 | 180 |
| Actomyosin | 335 | 339 | 100 | > 100 |

The intensity of fluorescence of actomyosin on denaturation weakly rose. These findings are in agreement with the paper of Theale and Weber [15] who detected a rise in quantum yield of fluorescence of globulins and a decrease in albumins on denaturation with urea.

It is to be hoped that the methods of luminescence investigation will prove to be useful in study of the different functional and physico-chemical properties of proteins.

## CONCLUSIONS

(1) An investigation has been made of the luminescence spectra of solutions of three aromatic amino acids and five proteins at room and low temperatures, down to —160°, in the 280–520 mμ wavelength region on excitation in the region from 240–280 mμ.

(2) The luminescence of the proteins was determined by the aromatic amino acids and in the proteins containing all three amino acids was determined exclusively by tryptophan, whereas in zein, not containing tryptophan, by tyrosine at room temperature and by tyrosine and phenylalanine at low temperature.

(3) Luminescence of the tryptophan in the protein molecule differs in spectrum from its luminescence in solution and approximates to that of tryptophan in frozen solution.

(4) The luminescence spectra of tryptophan residues vary in different proteins and undergo change on heat denaturation of the protein molecule.

We wish to thank A. N. Terenin and A. A. Krasnovskii for constant interest and assistance in the work.

*Translated by* A. CROZY

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VOL. 5 1960

# RELATION BETWEEN MOLECULAR MORPHOLOGY OF MACROMOLECULES OF DEOXYRIBONUCLEIC ACID AND THEIR RADIOSENSITIVITY (PROBLEM OF THE RADIOSENSITIVE AND RADIORESISTANT FORMS OF DNA)*

P. I. TSEITLIN, D. M. SPITKOVSKII and N. P. RIABCHENKO

Institute of Experimental Biology, U.S.S.R., Academy of Medical Sciences, Moscow

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VOL
5
196

IN our previous communications [1, 2] we noted the presence of two configurations for DNA, characteristic within their very nature of the molecules of deoxyribonucleic acid. These discrete configurations are the contracted and extended forms of the DNA molecule.

As has been noted [2] when various influences produce reversible contraction of DNA and the changed form of the latter is effectively contracted then it must be more resistant to external factors — by virtue of the change in configuration — than the original extended form of DNA. The question is naturally one of a reasonable force for the influence which must not yet lead to degradation of the DNA molecule. The same must also be true of DNA molecules of different molecular weight: DNA with $M \leqslant 1 \times 10^6$ must in the configuration sense be more stable than DNA with $M > 1 \times 10^6$ since DNA with $M \leqslant 1 \times 10^6$ is only to be found in the contracted state [2].

Thus, if we consider that the primary changes in the DNA molecule are due to a metastable state of its extended form, then we may assume that the change in DNA, for example, with relatively low doses of ionizing radiations, is caused by transition of DNA from the extended to the contracted configuration state and that by virtue of the change in the parameters of the molecular chains the contracted form of DNA must be more radioresistant.

Unfortunately, such an approach to the problem is unknown to us. Reference can only be made here to the views of Mitchell [3] that the different behaviour of radioresistant and radiosensitive tumours is possibly due to the fact that the former contain low polymerized DNA. In line with our ideas DNA of low molecular weight ($M \leqslant 1 \times 10^6$) is to be found in a configuration state similar to the contracted form of the high molecular weight DNA ($M > 1 \times 10^6$).

The purpose of the present investigation is to compare the configuration radiosensitivity of the extended and contracted forms of DNA. Reversible compaction of the

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DNA molecule can be effected by changing the pH of the medium [4–7]. However, as already noted [2], with the various methods giving reversible contraction of DNA the contracted form has a configuration such as that of the DNA bound with protein in nucleoproteins.

We have in fact compared the radiosensitivity of the extended form of DNA with the contracted form corresponding to the non-protein model of the nucleoprotein molecule. This is of great interest in that the nucleoprotein differs from DNA in its considerably greater radioresistance in the configuration sense and in the absence of after effect [8]. Elucidation of the problems posed above must also give definite indications as to whether the lowered radiosensitivity is due to inclusion of protein in DNP or to the configuration of the nucleic acid molecules forming part of the nucleoprotein and differing from the configuration of free DNA.

VOL. 5 1960

## METHODS

We used two preparations of calf thymus DNA obtained by the method of Mirsky and Pollister [9] with subsequent removal of protein by the method of Sevag [10]. The DNA concentration was determined by the method of Spirin [11] and the molecular weight (M) from the ratio proposed by Spitkovskii [12]. Sample No. 1: N/P $\approx$ 1·8; $M = 2{\cdot}8 \times 10^6$ and sample No. 2: N/P $\approx$ 1·8; $M = 3{\cdot}8 \times 10^6$.

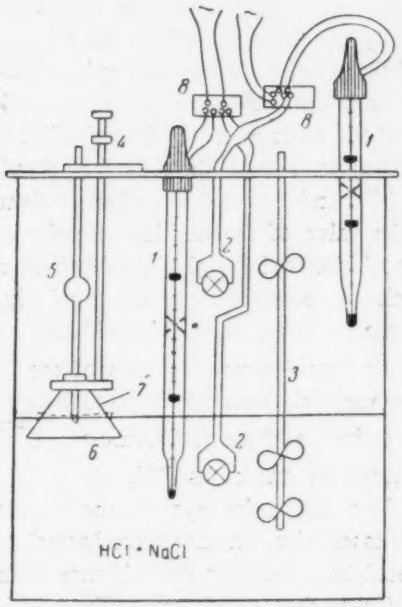


FIG. 1. Diagram of air-water thermostat for dialysis. (1) contact thermometers; (2) heaters; (3) stirrer; (4) microscrew; (5) viscometer; (6) cellophane membrane; (7) funnel for dialysis; (8) electromagnetic relays.

Irradiation was carried out from a RUT 60–20 unit (60 kV, 20 mA, 0·5 mm Al, focal distance 60 mm) at a temperature of +5°.

The viscosity was measured at a temperature of 25±0·05° in an Ostwald viscometer with a diameter and capillary length of 0·6 and 100 mm respectively. The initial DNA concentration in 0·2 M NaCl solution was 0·003 per cent.

The DNA molecules were contracted by dialysis of the 0·003 per cent solution of the nucleic acid in 0·2 M NaCl against dilute HCl in 0·2 M NaCl. Dialysis was run at a temperature of 25±0·05°. In order to avoid change in concentration and allow for the possibility of change in viscosity during dialysis we set up the combined thermostat, the layout of which is shown in Fig. 1. The temperature of the air and water was kept at 25±0·05° by means of air and water thermostats of the same capacity. The surfaces of the DNA solution for dialysis and of the external solution (the pH of which was adjusted stepwise to an end value of 2·60) were maintained at the same level by a microscrew.

## EXPERIMENTAL RESULTS AND DISCUSSION

Into the funnel for dialysis was poured 75 ml 0·003 per cent solution of the sodium salt of DNA in 0·2 M NaCl solution. As the pH was reduced samples of dialysate were withdrawn and placed in a refrigerator. When the pH of the DNA solution had reached 2·7, its viscosity had fallen to half and the final sample of DNA was withdrawn, the remainder of the solution being subjected to back dialysis against 0·2 M NaCl. Return of the value of the limiting viscosity, on back dialysis to the original value, served as control of the absence of depolymerization of DNA on acid-induced contraction. The DNA samples taken at the different pH values of the solution were irradiated with doses of 1670 and 2500 r. The effect of damage was assessed by the percentage decrease in the value of the limiting viscosity of the samples measured 15 min after irradiation in relation to the original pre-irradiation viscosity of the samples.

The experimental results are presented in Table 1 and Fig. 2 which gives the mean values for each fraction, the deviation from which did not exceed 5 per cent. Table 1 gives the mean square distances between the ends of the molecules ($\sqrt{\overline{R^2}}$) and the values ($a/b$) calculated for one of the possible configurations (an ellipsoid of revolution) of asymmetry of DNA arrived at from the formulae: $\sqrt{\overline{R^2}} = 376 \sqrt[3]{[\eta]^2}$ and $a/b = 47 \sqrt{[\eta]}$ previously presented by one of us [12].

Thus, as can be seen from the results reported above there are two forms of DNA macromolecule — the radiosensitive, extended configuration and the radioresistant, contracted form. It should be noted that the existence of the two forms of DNA is an experimental fact and that the quantitative differences between these two forms depend on the model chosen for calculation.

The acid-contracted configuration of DNA must, apparently, be more sensitive to the effect of radiation than the elongated one. In fact, on acidification of the DNA solution to pH 2·7, as is known, a considerable number of interspiral hydrogen bonds fuse in the DNA molecule are broken and from this point of view such a double helical structure is greatly attenuated as compared with the original one. However, according

TABLE 1. RADIOSENSITIVITY OF DNA MACROMOLECULES AS A FUNCTION OF THEIR CONFIGURATION

| Sample | M·10⁶ | pH | Original preparation | | | Irradiated preparation | | | | | | | Prep. No. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $\eta_{sp}/C$ (100 cm³/g) | $\sqrt{\overline{R^2}}$ (Å) | $a/b$ | Dose (r) | $\eta_{sp}/C$ (100 cm³/g) | $\sqrt{\overline{R^2}}$ (Å) | $a/b$ | Change $\eta_{sp}/C$ (%) | Change $\sqrt{\overline{R^2}}$ (%) | Change $a/b$ (%) | |
| No. 1 | 2·8 | 6·9 | 25·2 | 3220 | 236 | 1670 | 22·6 | 3010 | 224 | 10·0 | 6·5 | 5·0 | 1 |
| | | | | | | 2500 | 20·4 | 2810 | 212 | 19·0 | 12·7 | 10·2 | 2 |
| | | 2·9 | 23·1 | 3050 | 226 | 1670 | 19·8 | 2750 | 209 | 13·9 | 9·8 | 7·5 | 3 |
| | | | | | | 2500 | 17·5 | 2540 | 197 | 24·3 | 16·7 | 12·9 | 4 |
| | | 2·7 | 14·0 | 2190 | 176 | 1670 | 14·0 | 2190 | 176 | 0 | 0 | 0 | 5 |
| | | | | | | 2500 | 14·0 | 2190 | 176 | 0 | 0 | 0 | 6 |
| No. 2 | 3·8 | 6·9 | 33·8 | 3920 | 273 | 2500 | 27·8 | 3450 | 248 | 17·7 | 12·0 | 9·2 | 7 |
| | | 2·9 | 28·3 | 3500 | 250 | 2500 | 23·5 | 3080 | 218 | 17·0 | 12·0 | 8·9 | 8 |
| | | 2·7 | 18·7 | 2650 | 203 | 2500 | 18·7 | 2650 | 203 | 0 | 0 | 0 | 9 |

to indirect data, radiation causes rupture of the hydrogen bonds. It might have been expected that on irradiation of the acid-contracted form of DNA breakage of a small number of the remaining hydrogen bonds would occur and the effect of the fall in viscosity ought to be greater than on irradiation of extended DNA. Apparently, the resistance of the acid-contracted DNA to the given range of radiation doses indicates that the main damaging effect is associated with factors transforming DNA from a metastable, extended into a more stable, contracted configuration.

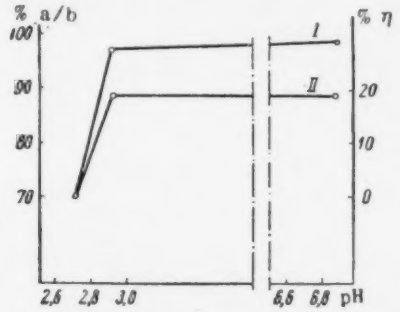


FIG. 2. Effect of DNA concentration on its radiosensitivity. (I) relative change in asymmetry ($a/b$) of unirradiated DNA molecule for various pH values; (II) relative change in characteristic viscosity of DNA on irradiation (dose 2500 r) in relation to pH of solution.

If we accept this thesis and bear in mind that on contraction of the molecule the maximum changes in asymmetry (considering the DNA molecule in the form of an ellipsoid of revolution), in viscosity and in the mean square distance account for about 25, 44 and 32 per cent respectively, it will be seen that the degree of configuration damage to the DNA macromolecule can be represented by relations of the type:

$$Q = Aq\%$$

where $q\%$, the percentage decline in viscosity, asymmetry or the mean square distance between the ends of the DNA molecule from external stimuli not producing depolymerization and $A$, a constant equal to: (1) 2·2 on assessment of configuration damage from viscosity; (2) 3·1 according to change in the value $\sqrt{\overline{R^2}}$; and (3) 4·0 according to change in $a/b$. Naturally, the suggested equation gives very similar values for the configuration damage irrespective of the molecular parameter selected. In this case the data given in Table 1 can be expressed in a different manner (Table 2).

As Table 2 shows the contracted form of DNA was much more resistant to the effect of ionizing radiations than the extended one. This fact is particularly significant since in our experimental arrangement increase in hydrogen ion concentration ought to have led to increased sensitivity of the preparation to the action of ionizing radiation — if we consider the effect of pH independently of the configuration changes in the DNA. However, it was found that the effect of acid in the pH range 6·9–2·9 with the DNA still in the extended state did not produce any fundamental changes in the high radiosensitivity of the DNA. A slight shift in the pH to 2·7 led to a sharp rise in radioresistance of the DNA (consideration of the value of ionization of definite groups of the nitrogenous bases of DNA responsible for the presence of the interspiral hydrogen bonds for given pH values should have produced the opposite of that described). Thus, the presence of radioresistant and radiosensitive forms of DNA is associated solely with the features of the configuration of the latter. This conclusion is confirmed by the absence of an after effect both in the contracted form of DNA and

TABLE 2. RADIOSENSITIVITY OF DNA MACROMOLECULES AS A FUNCTION OF THEIR CONFIGURATION

| Sample | Preparation No. | pH | Dose (r) | Percentage configuration damage |
|---|---|---|---|---|
| No. 1 | 1 | 6·9 | 1670 | 20 |
| | 2 | | 2500 | 40 |
| | 3 | 2·9 | 1670 | 30 |
| | 4 | | 2500 | 50 |
| | 5 | 2·7 | 1670 | 0 |
| | 6 | | 2500 | 0 |
| No. 2 | 7 | 6·9 | 2500 | 37 |
| | 8 | 2.9 | 2500 | 37 |
| | 9 | 2·7 | 2500 | 0 |

in the case of the nucleoprotein (in which DNA is in the contracted state) and by the sharply marked radioresistance of DNA with $M \leqslant 1 \times 10^6$. These facts form the subject of special communications [8, 13].

As pointed out above, we had earlier obtained results showing that the nucleoproteins in which DNA is in the contracted state are more radioresistant than the extended DNA. In the light of the material presented in this paper, the resistance of nucleoproteins to radiation treatment can be attributed not to the protective effect of the

protein but to the contraction on complex formation of the DNA molecule as a result of which it passes into a configuration resistant to external stimuli.


## SUMMARY

(1) The presence of radioresistant and radiosensitive forms of DNA associated with features of the configuration of the latter has been demonstrated.


*Translated by* A. CROZY

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* There is a translation into English of this work.

# CERTAIN FEATURES OF THE PROCESSES OF DISTURBANCE OF STRUCTURAL STABILITY OF SERUM PROTEINS IN MALIGNANT NEOPLASMS*


V. K. TKACH

Gor'kii State University, Kharkov


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IN a number of investigations aimed at studying the mechanism of development of malignant neoplasms and the properties of tumour tissue, it has been noted that in

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the serum proteins extracellular fluid and tissue proteins there occur different physicochemical and biochemical changes and processes suggestive of specific structural changes in the protein macromolecules caused by the tumour developing within the body.

A certain reflexion of these processes is provided by changes in some biophysical and physicochemical criteria and in properties characterizing the given medium, a study of which makes it possible to cast light on the specific features of the structural disturbances occurring within the serum proteins, nucleic compounds and tissues, of which as yet only isolated but highly important examples are known. Thus, for example, such changes include a decline in the value of the specific inductive capacitance [1, 2], change in the buffer capacity of the serum and the isoelectric point of proteins [2], disturbances in the form of the polarograph [3], disturbance of the character of water binding by tumour tissue [4], alteration in the processes of hydration of proteins [5–9], the peculiarities in the kinetics of loss of water by the proteins [10], the appearance of differences in structure and metabolism of nucleic compounds, normal protein and the proteins of the body affected with malignant tumour [11].

In addition, it has been shown [12] that under the influence of the products of a transplanted tumour the stability of the proteins in the tissues of the body is disturbed, the direction of these disturbances differing for the various stages of tumour development. Thus, in the first stages of development an increase is observed and at later stages a fall in protein stability. The mechanism of these phase changes of stability has not been discovered but it can be safely considered that it involves participation of changes in the molecular structure of proteins. Therefore, further study of the structural changes in the body proteins in the presence of a tumour is of great interest, in particular, elucidation of the molecular mechanisms of changes during development of malignant neoplasms, which is necessary for a deeper understanding of a number of features of this disease.

This paper gives an outline of material obtained in a study of certain processes caused by disturbance in the structural stability of the serum proteins of patients with breast cancer after introducing the proteins into a polar solvent (water).

In this case, the disturbances in the structural stability were manifest in protein dissociation and adsorption by the proteins of ions from the ambient medium. It was found that the intensity and duration of these processes varied with the severity of the disease and the efficacy of methods of treatment.

The preliminary investigations carried out earlier [13, 14] showed that a very convenient method for determining these disturbances was measurement of the high-frequency electrical conductivity of serum solutions in an optimal radio frequency range. Further investigations, the results of which are presented below have enabled us to work out a very sensitive method for recording structural disturbances by studying the kinetics of change in the temperature coefficients of the high-frequency electrical conductivity of the serum solutions. It is based on the premise that the mass of the charged protein particles in solution exceed by several orders of magnitude the mass of the background mineral ions in the solution of the test serum.

Here, solution of the differential equation of motion for an ion of mass $m$ in the high-frequency field $E = E_0 \sin \omega t$ with reference only to the force of friction of the ions and neglecting hydration of the ions, electrophoretic force and relaxation forces make it possible to find an approximated expression for the high-frequency electrical conductivity of a solution containing ions of given mass.

$$\sigma = \frac{Ne^2 K}{m(K^2+\omega^2)},$$

where $N$, number of ions per cm$^3$; $e$, charge on ion; $K$, coefficient of friction, $\omega$, field frequency.

In the presence in the solution of ions of differing mass, its total electrical conductivity can be found from the expression

$$\sigma = \frac{N_1 e_1^2 K_1}{m_1(K_1^2+\omega^2)} + \frac{N_2 e_2^2 K_2}{m_2(K_2^2+\omega_2)} + \cdots + \frac{N_n e_n^2 K_n}{m_n(K_m^2+\omega^2)}.$$

If, as a first approximation, we consider the serum solution with ionic background only as a two-component solution consisting of protein, mineral, OH and H ions, then obviously, by virtue of the difference in their mass, the greatest influence on the value of the electrical conductivity will be exerted by ions of small mass (for a sufficiently high frequency of the electrical field).

Therefore, ignoring the terms fiving the mass of the "large" protein ions, it may be considered that the electrical conductivity of the solution is determined in the main by the number of ions of smaller mass;

$$\sigma = \frac{Ne^2 K}{m(K^2+\omega^2)}$$

(symbols $N$, $e$, $K$, $m$, now refer only to the ions of small mass).

If we assume that the friction of the ions is proportional to their concentration in solution then it is always possible to select conditions in which $\omega \gg K$. Then the last equation assumes the form:

$$\sigma = \frac{Ne^2 K}{m\omega^2}.$$

This shows that in the region of low ionic concentrations, the high-frequency electrical conductivity of the solution (for a given but sufficiently high frequency) will rise with rising ionic concentration and conversely, fall with decreasing concentrations.

This fact is sufficiently well confirmed experimentally for ions of concentrations from $5 \times 10^{-4}$ to $25 \times 10^{-4}$ $N$ and for a frequency of the electrical field of $7{\cdot}5$–$8 \times 10^6$ c/s. Therefore, if the test protein undergoes structural changes accompanied by dissociation in solution with the formation of small ionized fractions, this will produce an increase in the electrical conductivity of the solution. Conversely, development in the proteins of structural changes accompanied by increased adsorptive powers in

relation to finely dispersed ions will produce reduction in their concentration in the solution and, consequently, a reduction in electrical conductivity. In the study of the kinetics of these processes it is not at all essential to determine absolute values of the electrical conductivity since methodologically it is simpler to measure the proportional temperature coefficients of high-frequency electrical conductivity characterizing its relative changes. The measurements were made with the serum of venous blood, 0·6 $cm^3$ was diluted in double distilled water in a ratio of 0·6:10. The first measurement was made 30 min after taking the blood with subsequent measurements after 1·5, 3, 4 hr, etc.

For the normal physiological state of the body the serum proteins in solution have a period of structural stability — "the temporal zone of stability" — which is determined by the fact that repeat measurements (at the indicated intervals of time) of the temperature coefficients of high-frequency electrical conductivity (t.c.h.f.e.c.) or after 2–3 and even 4 hr give unchanged values. In different individuals this value may vary but the value obtained in the first measurement always remains unchanged in the course of the following 1·5–2 hr, i.e. the difference in the t.c.h.f.e.c. values was zero, or changed within the limits of error of the measuring apparatus. In precisely the same way the duration of the period of stability varied within certain limits but was not less than 1·5 hr.

In order to shorten the time taken by the investigation it is not necessary to determine the entire time course but merely two successive measurements of the same serum 30 min and 1·5 hr after taking the blood. An example of the t.c.h.f.e.c. values and their differences for two such measurements of the same serum of nominally healthy individuals is provided by the data in Table 1. The differences of $\pm 100$ arbitrary units shown in Table 1 lie within the limits of error of the measuring arrangements.

TABLE 1. VALUES, IN ARBITRARY UNITS, OF TEMPERATURE COEFFICIENTS OF HIGH-FREQUENCY ELECTRICAL CONDUCTIVITY ($H$) OF SOLUTIONS OF SERUM OF NOMINALLY HEALTHY INDIVIDUALS

| Surname | $H_1$ after 30 min | $H_2$ after 1·5 hr | $\Delta H$ | Surname | $H_1$ after 30 min | $H_2$ after 1·5 hr | $\Delta H$ |
|---|---|---|---|---|---|---|---|
| L-g | 4300 | 4300 | 0 | M-a | 2000 | 2100 | +100 |
| V-o | 3400 | 3500 | +100 | Kh-ch | 4000 | 3900 | —100 |
| D-v | 3000 | 3000 | 0 | N-a | 3000 | 3000 | 0 |
| Sh-i | 2400 | 2400 | 0 | N-o | 2900 | 2900 | 0 |
| G-o | 1700 | 1700 | 0 | K-o | 4000 | 4000 | 0 |
| S-v | 2000 | 2000 | 0 | P-i | 3800 | 3800 | 0 |
| Sh-n | 2200 | 2100 | —100 | S-o | 6600 | 6600 | 0 |
| Kh-ia | 2600 | 2500 | —100 | N-i | 4500 | 4500 | 0 |
| A-a | 2200 | 2300 | +100 | K-a | 3800 | 3800 | 0 |
| Kh-ia | 1800 | 1800 | 0 | S-o | 3200 | 3200 | 0 |

In investigations of the serum of patients with malignant neoplasms of different origin an entirely different picture was observed. The temporal zone of stability was greatly shortened reaching 20–30 min or was practically absent. Repeat measurements

TABLE 2. VALUES, IN ARBITRARY UNITS, OF TEMPERATURE COEFFICIENTS OF HIGH-FREQUENCY ELECTRICAL CONDUCTIVITY ($H$) OF SOLUTIONS OF SERUM OF PATIENTS WITH DIFFERENT STAGES OF BREAST CANCER (BEFORE TREATMENT)

| Diagnosis and stage | $H_1$ after 30 min | $H_2$ after 1·5 hr | $\Delta H$ | Diagnosis and stage | $H_1$ after 30 min | $H_2$ after 1·5 hr | $\Delta H$ |
|---|---|---|---|---|---|---|---|
| Mammary carcinoma Stage IV | 6600 | 7200 | +600 | Mammary carcinoma Stage I | 3600 | 4400 | +800 |
| ,, II | 10,800 | 6000 | —4800 | ,, I | 1100 | 2600 | +1500 |
| ,, II | 6600 | 6200 | —400 | ,, II | 8700 | 5500 | —3200 |
| ,, II | 4200 | 8300 | +4100 | ,, II | 1500 | 5300 | +3800 |
| ,, III | 7700 | 8000 | +300 | ,, II | 6800 | 7700 | +900 |
| ,, II | 2600 | 6000 | +3400 | ,, III | 1000 | 5800 | —4200 |
| ,, II | 11,000 | 10,600 | —400 | ,, IV | 2800 | 3600 | +800 |
| ,, II | 6300 | 8900 | +2600 | ,, II | 700 | 1200 | +500 |
| ,, I | 1700 | 2300 | +600 | ,, II | 1300 | 1000 | —300 |
| ,, III | 7800 | 8800 | +1000 | ,, I | 5000 | 3800 | —1200 |
| ,, IV | 2800 | 4000 | +1200 | ,, II | 9000 | 5600 | —3400 |
| ,, II | 2400 | 3600 | +1200 | ,, III | 2800 | 1400 | —1400 |
| ,, II | 2400 | 3600 | +1200 | ,, I | 1900 | 2300 | +400 |
| ,, II | 3800 | 5200 | +1400 | ,, II | 3800 | 6400 | +2600 |
| ,, III | 3900 | 3500 | —400 | ,, II | 6600 | 3600 | —3000 |
| ,, II | 4400 | 3600 | —800 | ,, II | 4400 | 4000 | —400 |
| ,, II | 3200 | 2300 | —900 | ,, III | 5200 | 4700 | —500 |
| ,, II | 1800 | 1000 | —800 | ,, III | 5400 | 3700 | —1700 |
| ,, III | 6000 | 6400 | +400 | ,, II | 1600 | 800 | —800 |
| ,, II | 2400 | 1200 | —1200 | ,, II | 1600 | 1100 | —500 |
| ,, III | 3600 | 4800 | +1200 | ,, II | 800 | 400 | —400 |
| ,, II | 2600 | 1600 | —1000 | ,, II | 2600 | 1300 | —1300 |
| ,, III | 2500 | 4400 | +1900 | ,, II | 4400 | 4000 | —400 |
| ,, I | 5100 | 4500 | —600 | ,, IV | 4000 | 6000 | +2000 |
| ,, III | 2400 | 3600 | +1200 | ,, II | 3200 | 1700 | —1500 |
| ,, I | 4400 | 4800 | +400 | ,, II | 2300 | 1100 | —1200 |
| ,, I | 4400 | 4800 | +400 | ,, II | 2300 | 1600 | —700 |
| ,, II | 2900 | 5200 | +2300 | ,, II | 1500 | 700 | —800 |
| ,, III | 900 | 1900 | +1000 | ,, II | 6600 | 6000 | —600 |
| ,, II | 1300 | 2000 | +700 | ,, II | 4200 | 4000 | —200 |
| ,, II | 5200 | 3600 | —1600 | ,, II | 2000 | 1500 | —500 |
| ,, II | 4400 | 8800 | +4400 | ,, III | 5600 | 5800 | +200 |
| ,, IV | 2600 | 5900 | +3300 | ,, II | 1900 | 1500 | —400 |
| ,, II | 3600 | 3200 | —400 | ,, II | 1400 | 900 | —500 |
| ,, IV | 4400 | 5600 | +1200 | ,, II | 4200 | 4400 | +200 |
| ,, II | 6100 | 3000 | —3100 | ,, II | 900 | 2000 | +1100 |
| ,, II | 3400 | 3600 | +200 | ,, I | 4500 | 5000 | +500 |
| ,, II | 4200 | 3400 | —800 | ,, II | 4200 | 4000 | —200 |
| ,, II | 9800 | 6000 | —3800 | ,, III | 4000 | 1000 | —3000 |
| ,, IV | 3000 | 4600 | +1600 | ,, II | 3200 | 2600 | —600 |
| ,, IV | 3200 | 4000 | +800 | ,, II | 2400 | 2000 | —400 |

VOL. 5 1960

of the t.c.h.f.e.c. of serum solutions of these patients showed a rise from measurement to measurement or, conversely, a reduction. The first conclusion to be drawn from this is that the structural stability of the serum proteins of these patients is low and this is manifest in a reduction in the temporal zone of stability or even in its absence. The disturbances in stability observed are of a dual character. In some cases the disturbances in the structural stability were accompanied principally by the development of processes of dissociation (in this case the difference between two measurements of the t.c.h.f.e.c. was positive), whereas in the other cases there was a development chiefly of the adsorption processes in which there must occur a fall in the number of small dispersed ions in test solution with resulting reduction in the t.c.h.f.e.c. value from one measurement to the next (in this case the difference between two measurements of the t.c.h.f.e.c. was negative).

Such a difference in the processes of disturbance of protein stability was also observed for the same clinically established stage of the disease. This can be seen on inspection of some of the results of the investigations given in Table 2 and obtained on measurement of the t.c.h.f.e.c. of the serum solution of patients with breast cancer in various stages.

Analysis of the results of the investigations show that for the first three stages of the disease it was impossible to observe a uniform disturbance of structural stability. For clinically identical stages of the disease a rise and fall in the t.c.h.f.e.c. could be recorded and only for the fourth stage of the disease was there a rise in the t.c.h.f.e.c. observed from one measurement to the next, indicating that in these cases development chiefly of the dissociation processes occurs.

Statistical treatment of the results obtained on measurement of serum solutions of patients in the first, second and third stages of the disease, nevertheless brought out a definite relationship between the processes of dissociation and adsorption. Thus, for stage III only in about 60 per cent of the patients was there development chiefly of the dissociation processes while in the other 40 per cent of the patients the disturbances were accompanied by adsorption processes. In the patients of stages I–II, development of the dissociation processes was observed only in 36 per cent, whereas in the remaining 64 per cent of the patients disturbances in structural stability accompanied by development of adsorption processes predominated. These findings allow us to assume that in the development of malignant neoplasms the structural stability of the serum proteins starts to decline and the clinically milder stages of the disease are accompanied chiefly by the development of adsorption activity of the serum proteins in solution in relation to the small dispersed ions, whereas the graver stages of the disease are accompanied in the main by the development of protein dissociation. In order to judge how far the changes described are specific for malignant neoplasms further comprehensive investigations of sera of other pathological types are necessary.

The findings presented show that the protein structures of the serum in the development of a given type of disease undergo changes during development of the disease indicative of different molecular properties of the proteins of the diseased body. This underlines the urgent need for wider and more comprehensive investigation of the disturbances in structural stability of serum proteins in various conditions of the body.

VOL
5
196

## SUMMARY

(1) Measurement of the temperature coefficients of high-frequency electrical conductivity of solutions of serum of patients with breast cancer have been made to investigate the processes of disturbance in structural stability of proteins.

(2) It was found that for virtually healthy individuals structural stability of the serum proteins in solution is observed for 2–3 hr as shown by constant values of the t.c.h.f.e.c. obtained in successive measurements of the same serum.

(3) In the development in the body of a malignant tumour the temporal zone of stability is greatly reduced or is completely absent and processes of protein dissociation and ionic adsorption develop.

(4) In the more advanced stages of the disease dissociation processes predominate while for stages I–II there is chiefly development of ionic adsorptive processes.


*Translated by* A. CROZY

VOL. 5 1960

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# ELECTROPHYSIOLOGICAL INVESTIGATION OF THE THRESHOLD SENSITIVITY OF THE DARK-ADAPTED EYE OF THE FROG AND THE INFLUENCE ON IT OF RED AND BLUE LIGHT*

L. P. KUZNETSOVA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
5
196

THE colorimetric experiments of Bongard [1] established that the frog possesses colour vision. Its colour vision is dichromatic and due to functioning of two receptors in the retina — daylight associated with the work of the cones and twilight associated with the work of the rods. As these experiments showed both receptors function for a long time in the isolated eye so that it is possible to study the properties of these receptors and their interaction in the retina in various conditions of adaptation of the eye. In order to study the work of the receptors frequent use is made of their threshold sensitivity which, as is known, is reduced on light adaptation and raised on dark adaptation. Such changes in threshold sensitivity of the frog eye have been investigated in a number of studies by the electrophysiological technique of recording the potentials formed in the eye on illumination.

For example, in the experiments of Riggs [2] and Granit, Munsterhjelm and Zewi [3] changes in the threshold sensitivity of the frog eye during dark adaptation were investigated from the changes in amplitude of the *b* wave of the electroretinogram. In the experiments of Riggs a rise in threshold sensitivity to flashes of white light started from the first minutes of dark adaptation. In the experiments of Granit and others a rise in the threshold of sensitivity to light of $\lambda = 550$ mμ began only 1 hr after the start of dark adaptation.

In the work of Hartline [4] the technique of recording the neurogram of the isolated fibre of the optic nerve of the frog was applied. In the fibres dissected from the papilla of the optic nerve it was established that after 30 min dark adaptation the threshold of the isolated fibre to light stimulation fell by a factor of 100–1000. More detailed investigation of the thresholds of the same fibre could not be carried out because of the rapid dying of the fibre at the supporting electrode.

In the later work of Granit [5] and Barlow [6] the thresholds of the individual elements of the retina were recorded by means of microelectrodes. In Granit's experiments, change in threshold sensitivity of the individual elements during dark adaptation lasting 40 min varied for different colour stimuli. The threshold sensitivity

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to red ($\lambda = 650$ m$\mu$) was raised slightly only in the first minutes of dark adaptation and did not subsequently change. Threshold sensitivity to green ($\lambda = 500$ m$\mu$) and blue ($\lambda = 450$ m$\mu$) was considerably increased throughout the entire period of dark adaptation.

In the work of Barlow the threshold of the "off-element" after 1 hr dark adaptation fell about 70 times. The thresholds to blue were reduced in the dark much more than the thresholds to red but no quantitative data on the rate and size of change in the thresholds to the applied colour stimuli are given.

A more convenient technique for prolonged experiments on the threshold sensitivity of the animal eye is the method of recording the neurogram for the whole optic nerve since the integral nerve in contact with the electrode does not die off and retains conductivity for a long period. This technique was used in the present work in order to determine the threshold sensitivity of twilight and daylight receptors of the frog eye during dark adaptation.

OL.
5
960

The main purpose of the work was to study the interaction of the two receptors functioning in the retina of the frog eye; an investigation was made of the effect of red light, exciting chiefly the cones, on the threshold sensitivity of the twilight receptor and of the effect of blue light, exciting chiefly the rods, on the threshold sensitivity of the daylight receptor. The investigation by means of electrophysiological techniques of the effect of red light on the threshold sensitivity of the twilight receptor of the dark-adapted eye is of special interest, it seems to us, in view of the hitherto unsolved problem of its stimulatory effect on the threshold sensitivity of the dark-adapted human eye [7–11].

With respect to the frog eye such observations have been made, in part, in the experiments of Granit, Holmberg and Zewi [12]. In the enucleated dark-adapted eye a change in the height of the *b* wave of the e.r.g. was recorded. The change was produced by a test flash of light ($\lambda = 550$ m$\mu$) after stimulating the eye for 5 min with equi-energetic monochromatic radiations. It was established that during the 6 min of the investigation, the electrical response to test light was depressed after any of the irradiations applied and that it was most strongly depressed after irradiation by light from the long wave region of the spectrum. Unfortunately, in these experiments a method was used which is of little use for investigating dark adaptation. In order to determine the height of the *b* wave at 0·7 mV, taken in these experiments as 100, it was necessary to deliver to the eye test flash intensities greatly in excess of the threshold; thereby the state of dark adaptation of the retinal test site was disturbed. In addition, the investigation of the change in electrical response after colour illumination of the eye was limited to merely 6 min and its further course of restoration remained unexplained.

## METHODS

The experiments were performed on the isolated eyes of *Rana temporaria* and *R. radibunda*. The eye was isolated together with the optic nerve and a portion of the brain, the anterior media were removed and the preparation placed in a humid chamber,

During the experiment the eye was kept in a dark screened room. The nerve was attached to two platinum electrodes connected, through a preliminary two-stage triode amplifier with a low level of inherent noise, to a 28-IM a.c. amplifier. Changes in the potentials occurring in the nerve on illuminating the eye were recorded visually by the pointer of a microvoltmeter connected to the amplifier.

In order to determine the threshold sensitivity during dark adaptation of the eye it was periodically illuminated with monochromatic light from an adaptometer-monochromator (Gurtovoi and Kravkov [13]). The light from the monochromator was directed to the screened room and reflected by means of a mirror onto the preparation. The intensity of the monochromatic radiations delivered to the eye was varied by using a photo-wedge with a logarithmic scale placed in front of the entry slit of the monochromator. In front of the illuminating lamp of the monochromator, the voltage of which was kept constant, there was an opaque slide to prevent light impinging on the eye in the intervals between the threshold measurements.

In the investigations on the threshold sensitivity of the twilight receptor monochromatic light with $\lambda \approx 470$ mμ was used in some experiments and with $\lambda \approx 540$ mμ in others — acting chiefly on the rods. In investigating the threshold sensitivity of the daylight receptor we used red light with $\lambda > 640$ mμ, acting chiefly on the cones.

The experiment was begun after preliminary light adaptation. In some experiments for this purpose we used light with a brightness on the retina of about 700 lx, in others about 5000–8000 lx. The light adaptation lasted from 10 to 20 min.

After preliminary light adaptation, the preparation was left in the dark and the thresholds measured from the total neurogram of the optic nerve after definite time intervals. For this purpose the wedge was slowly shifted from the position of its maximum density to a position of lower density and the opaque slide in front of the monochromator lamp periodically moved aside and replaced after 2–3 sec. The wedge division was noted at which turning on or off the light first produced a deflexion of the pointer on the microvoltmeter. The intensity of light transmitted by the wedge, corresponding to the division of the wedge, was taken as the threshold.

Measurements of the thresholds were carried out every 5–10 min during the entire period of dark adaptation alternating between the long wave and short wave light. The period of illuminating the preparations for the threshold determinations was 1–2 sec.

Alongside the main experiments with the isolated eye, for the sake of comparison some tests were carried out on the threshold sensitivity of the eye of the intact frog. In these experiments the frog was rendered motionless by injection of 1 ml 0·3% diplacin. The skull was opened from the side of the palate and a segment of optic nerve between the chiasma and the eye dissected out. The dissection was carried out without damage to the blood vessels in order to maintain the normal blood supply to the eye and optic nerve. A platinum electrode was placed under the exposed segment of the nerve slightly raising it. A fine glass tube was inserted under the raised nerve for siphoning off the interstitial fluid ensuring constant recording conditions. A second electrode was

pushed into the mucous membrane of the palate. The conditions of recording the impulses were the same as for the eye preparations.

For the light adaptation experiments on the intact frog we used a 100 W reflector lamp about 50 cm away from the frog eye. In these experiments, the threshold sensitivity of the eye to green light with $\lambda \approx 540$ mμ was investigated, no experiments were carried out with red light.

In the experiments with red and blue light a special illuminating lamp (30 W, 6 V) was used placed in a screened chamber above the preparation at a distance of 30 cm. The voltage of the lamp was kept at 6, 4 or 2 V which gave a variable intensity of light. In the case of red light, we placed in front of the illuminating lamp a 2 mm thick RG5 Schott filter transmitting rays longer than 640 mμ. For blue light we used a 2 mm thick BG12 Schott filter transmitting rays with $\lambda \approx 470$ mμ.

VOL. 5 1960

In a number of experiments the light was delivered by means of the blue or red radiations from the monochromator-adaptometer with the wedge fixed in its position of mininum density. The light was delivered after establishing a steady level of threshold sensitivity, i.e. after a few hours of dark adaptation. The duration of the lights was 5–15 min. The threshold measurements were made by the usual methods.

The experimental results are shown in the form of curves in which the time of dark adaptation in min is given along the abscissa and the ordinates are the log relative threshold sensitivities.

## RESULTS

The experiments on the isolated eyes of *R. radibunda* and *R. temporaria* established marked differences in the twilight and daylight receptors of the frog eye both in relation to their absolute thresholds and the rate of adaptation, which makes it possible to investigate the work of each separately.

In all the experiments a rapid rise in the threshold sensitivity to blue and green was observed from the first minutes of dark adaptation, slowing down the longer the eye was kept in the dark. The steady level of threshold sensitivity for blue and green was reached after a few hours dark adaptation (from 2 to 5 hr in individual experiments).

The threshold sensitivity to red light also changed from the first minutes of dark adaptation but the character of these changes varied in the different experiments. In some, threshold sensitivity to red was raised from the first minutes of darkness, whereas in others carried out by the same technique it diminished in the first 60–90 min and then started to rise. Sometimes in such experiments a fall in threshold sensitivity was preceded by a brief though slight rise in sensitivity. The steady level of the thresholds to red was reached at the same time as the steady level to blue and green.

The magnitude and rate of change in thresholds varied in the different experiments and in repeat variants for the same eye but their characteristics for red and blue persisted in all the experiments.

In Fig. 1 (a) and (b) the results are given of two experiments on the isolated eye of *R. temporaria* which are also typical of those on *R. radibunda*. Preliminary light

adaptation in these experiments was 700 lx. As the curves show, in both experiments the sensitivity to blue (continuous line with small circles) at first rose rapidly and then more slowly. The threshold sensitivity to red (dotted line with crosses) in one experiment (a) gradually rose during the entire period of dark adaptation as from the first minutes. In the other experiment (b) the threshold sensitivity to red after a slight rise in the first minutes of dark adaptation fell and only after about 60 min did it start to rise.

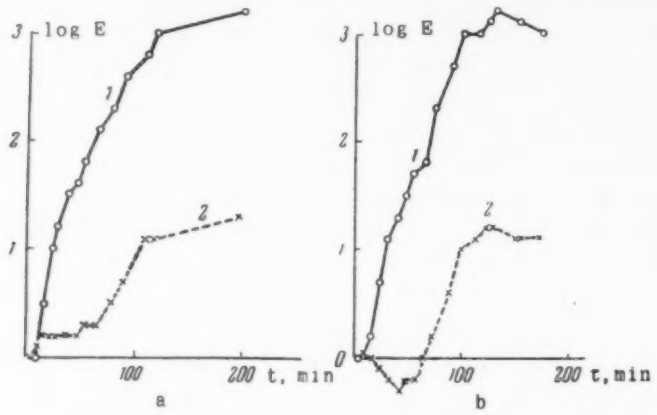


FIG. 1. Curves of change in threshold sensitivity of isolated eye of *R. temporaria*: (1) to blue (λ 470 mμ) and (2) to red (λ 640–680 mμ). Preliminary light adaptation to 700 lx. (a) experiment showing rise in threshold sensitivity to red from start of dark adaptation; (b) experiment showing decrease in threshold sensitivity to red in first hour of dark adaptation with subsequent rise. Abscissa, time of dark adaptation; ordinates, log relative sensitivities.

The magnitude of the threshold sensitivity after 3-hr adaptation for red exceeded the original level by about 10–20 times and for blue by about 2000 times.

Similar results were obtained in experiments with preliminary light adaptation with 5000–8000 lx. The experimental results on the intact frog shown in Fig. 2 (a) and (b) were obtained on the same frog on different days. In one of the experiments (a) the threshold curve was obtained for green light during 140 min dark adaptation, the other (b) during 60 min. The curves show that the threshold sensitivity to green rose from the first minutes of dark adaptation, as in the experiments on the isolated eye. After dark adaptation for 1 hr the threshold sensitivity to green had increased 180 times and this closely coincides with the value of the increase in the threshold sensitivity to blue for the corresponding period of dark adaptation of the isolated eye (Fig. 1).

The results of the experiments with lights are shown in Fig. 3 (a) and (b) in the form of curves obtained for the isolated eye of *R. radibunda*. In one experiment (a) preliminary light adaptation was with 700 lx, in the other (b) 5000–8000 lx. In the first experiment blue light was delivered from a monochromator for 5 min and the subsequent three lights — red light — from the illuminating lamp at a voltage of 2, 4 and 6 V. In the second experiment (b) the blue light was obtained by means of

the illuminating lamp at a voltage of 2 and 4 V. As is clear from the typical curves for this series of experiments, the blue light from the monochromator did not exert any appreciable effect on the threshold sensitivity of the eye. The blue light from the illuminating lamp produced only a fall in the threshold sensitivity both to red and blue with a subsequent return to the original level. The red light used did not exert

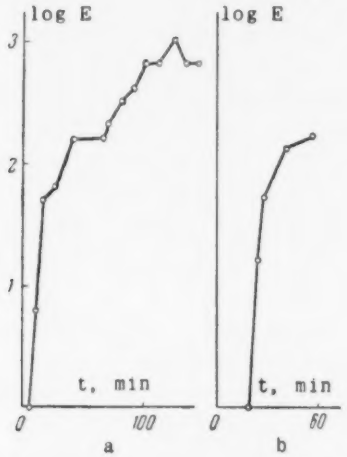


FIG. 2. Curves of change in threshold sensitivity of the eye of live frog *R. radibunda*, rendered motionless with an injection of diplacin, to green light with $\lambda \approx 540$ mμ. Preliminary light adaptation to 5000–8000 lx (a) first experiment; (b) second experiment on same frog.

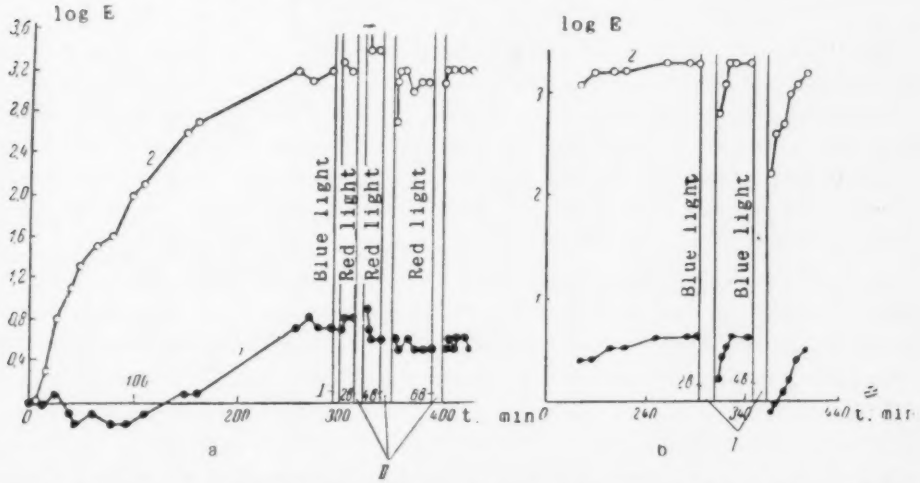


FIG. 3. Effect of red and blue light on threshold sensitivity of darkadapted eye of *R. radibunda* to red light. (1) $\lambda >$ 640 mμ; (2) $\lambda$ 470 mμ. (a) experiment with preliminary adaptation to 700 lx and blue light (I) from monochromator and (II) with red light from illuminating lamp with voltage of 2, 4 and 6 V; (b) experiment with preliminary light adaptation to 5000–8000 lx and blue light from illuminating lamp with voltage of 2 and 4 V.

any stimulatory effect on the threshold sensitivity of the dark adapted eye and the scatter of results was within the limits of experimental error.

More than 50 experiments were carried out on isolated eyes and about ten on the eyes of the live frogs.

## DISCUSSION OF RESULTS

Application of the method of recording the thresholds from the total neurogram of the intact optic nerve made it possible to investigate changes in the threshold sensitivity of the frog eye during a long period of dark adaptation.

The results obtained on the rate and magnitude of the increase in threshold sensitivity of the eye to short wave light stimuli during dark adaptation are essentially in agreement with those of the workers mentioned above. These findings were similar both for the isolated eye and for the eye of the intact frog. The concurrence of the results may be taken as evidence that change in the threshold sensitivity in the isolated eye corresponds to the change in the eye of the live frog and is not the product of pathological changes in the preparation.

VOL
5
196

Somewhat unexpected were the results on the changes in threshold sensitivity to red. It is commonly assumed that the thresholds to red fall only in the first minutes of dark adaptation and scarcely change at all on keeping the eye for a further period in the dark. In our experiments a rise in threshold sensitivity to red was observed during the entire period of dark adaptation which terminated simultaneously with the achievement of the steady level of threshold sensitivity to blue and green. However, in some experiments the threshold sensitivity to red was lowered in the first minutes of dark adaptation and then started to rise until the steady level was reached. Such a decline in the threshold sensitivity to red in the initial stage of dark adaptation has already been observed in relation to the frog eye, in the electrophysiological experiments of Therman [14], and to the pigeon eye in Blough's experiments on the conditioned reflex method [15, 16]. These workers consider that a probable cause of such results is inhibition of cone activity, which is greatly enhanced in conditions of the initial period of dark adaptation, through the activity of the rods. The absence of convincing proof of the presence of a depressant effect by the rods on the activity of the cones seems in our view to leave this problem still unsolved.

Nor is it clear whether the considerable rise in threshold sensitivity to red light during a prolonged period of dark adaptation, as observed in our experiments, was the result of the rise in the sensitivity of the cone receptor or the result of the very high sensitivity of the twilight receptor rendering it sensitive in the longwave region.

The results of the experiments with various lights do not bear out our expectations. On the basis of the findings of some work on man [7] which established a stimulatory effect of red light, we hoped by means of the electrophysiological technique to obtain a similar stimulatory effect of red light on the threshold sensitivity of the rod receptor of the frog eye. However, as our experiments show, the different intensities of red light used in our experiments — from weak to intensities exceeding the threshold by some hundreds of times — did not stimulate the threshold activity of the rod receptor.

Our experiments do not confirm the results of the experiments of Granit [12] — that the highest inhibitory effect on the threshold sensitivity of the dark adapted eye to short wave stimuli occurred after illumination of the eye with red light. In our experiments, blue light temporarily reduced the threshold sensitivity of the eye both to red and blue and red light practically did not alter it.

## SUMMARY

(1) The isolated eye of the frog retains the ability to change its threshold sensitivity during prolonged dark adaptation.

(2) The threshold sensitivity to blue and green (response of the twilight receptor) was increased in the first minutes of dark adaptation, the increase coming to an end only after the eye had been in the dark for several hours. The rapid rise in threshold sensitivity in the first hour of dark adaptation was slowed down during subsequent adaptation.

(3) Threshold sensitivity to red (response of daylight receptor) was raised in a number of experiments from the first minutes of dark adaptation, in others its rise was preceded by a fall in threshold sensitivity for the first 60–90 min.

(4) The threshold sensitivity to blue and green at the moment of establishing the steady level in the dark exceeded the original value some hundreeds and even thousands of times. The threshold sensitivity to red during this time had increased only some tens of times.

(5) A close correspondence was observed between the relative change in the threshold sensitivity of the isolated eye and the eye of the intact frog to short wave light stimulus in the first hour of dark adaptation.

(6) Red and blue lights did not exert any stimulatory effect on the threshold sensitivity of the dark-adapted frog eye.

*Translated by* A. Crozy

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VOL. 5 1960

# DISCRIMINATION OF A USEFUL SIGNAL BY THE AUDITORY ANALYSER—I. POTENTIALS FROM THE ELEMENTS OF THE FROG AUDITORY NERVE*

G. V. GLEKIN and G. M. ERDMAN

Institute of Acoustics, U.S.S.R. Academy of Sciences, Moscow

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ONE of the important problems in constructing a theory of hearing is that of the link between the mechanical receptor part of the auditory analyser and the conducting nerve pathways.

The investigations in this field have been made on animals with a quite highly developed, complex and minutely specialized organ of corti. Only a few papers deal with the processes of transmission of information in animals with primitive organization of the inner ear [1, 2].

The observations of Galambos and Davis [3], Tasaki [4, 5] and in recent years Sumi, Katsuki, Uchiyama, Maruyama and Watanabe [6–8] have established the patterns of discrimination of characteristic frequencies in the mammalian auditory nerve. The most plausible assumption is that the process of discrimination of any frequency in the nerve conducting pathway is closely related to the mechanical processes observed at the membranes of the organ of corti. It is thus important to establish whether the primitive character of the auditory receptor of amphibia is reflected in the conduction and discrimination of a useful signal in the auditory analyser.

It may be conceived that in the case of mammals the process of discrimination of a useful signal proceeds as a gradual — at the levels of the auditory tract — discrimination of a definite frequency bearing useful information provided that the primary discrimination of this frequency comes about as a regional maximum of mechanical vibrations on the membranes of the organ of corti associated with deformation of the fluids of the cochlea and of the hair-cells [9]. The inner ear of the frog is not constituted as in mammals, i.e. membranes are absent from the bladder-shaped sacculus of the frog and are replaced by a so-called basila papilla which morphologists (Shmal'gauzen, *et al.*) see as a rudimentary basila membrane. Consequently, in relation to the frog there are no grounds for assuming the possibility of initial discrimination of frequency as a regional maximum of mechanical vibrations.

The problem was to investigate whether discrimination of a definite frequency occurs in the conducting part of the frog auditory analyser in a manner similar to that observed in the conducting pathways of the mammalian auditory analyser.

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Preparing the specimen, so as to record from the individual neurones of the auditory tract of the frog, with the aim of exposing the nerve and the sacculus with complete retention of auditory function was quite a complicated matter. We have not found a description of this dissection in the literature.

As Shmal'gauzen writes [10] in an article on the auditory apparatus of amphibia; "the sound vibrations of the air are perceived in amphibia by an elastic membrane of fairly large surface — the tympanic membrane — and are transmitted by means of the auditory ossicle — the columella auris — to a much smaller membrane of the oval window of the auditory capsule and then to the perilymph and the endolymph of the inner ear". Further, Shmal'gauzen points out: "the ossicle mechanically transmits the vibrations of the tympanic membrane to the oval window".

Consequently, during dissection it is important to preserve the ossicle of the middle ear in order not to disturb normal transmission of sound vibrations to the oval window.

The anatomical structure of the frog auditory analyser is such that this is practicable only by approaching the sacculus from below, i.e. from the oral cavity. The operation was performed on the isolated head of the frog *Rana radibunda.*

The head was isolated in order to prevent the lower jaw with the tongue from impeding the dissection: the skin with the occipital part of the head was cut through. The lower surface of the bones of the base of the skull was exposed and, using a binocular dissecting lens, pieces of bones were carefully snipped off by transverse cuts. Firstly, the solid bones of the base of the skull were removed and the dorsal side of the brain exposed, then the lateral bones and cartilages were snipped through and the sacculus exposed. Next, the bones covering the course of the VIII$^{th}$ nerve and its branches were carefully removed. A diagram of the structure of the frog auditory apparatus is shown in Fig. 1.

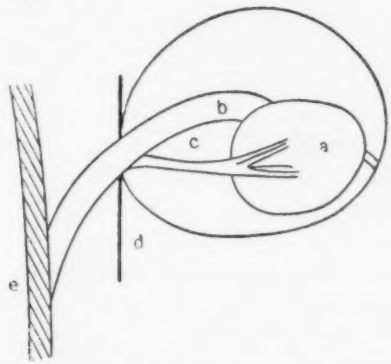


FIG. 1. Diagrammatic structure of frog auditory nerve tract. (a) sacculus; (b) trunk of VIII$^{th}$ nerve; (c) saccular branch; (d) skull bone; (e) brain.

When all the bony teguments were removed, we could easily trace the course of the nerve from the surface of the sacculus to the surface of the brain and distinguish the saccular branch with the fan-shaped diverging fibres connecting the VIII$^{th}$ nerve and the receptor part of the auditory analyser.

However, in such a form the nerve conducting tract of the frog is still not ready for receiving microelectrodes. It was necessary to remove the fairly solid and elastic membranes covering the sacculus and the saccular branch. This, the most delicate part of the operation, was carried out by means of a micromanipulator designed by us in the Institute of Acoustics of the U.S.S.R. Academy of Sciences in collaboration with engineer Z. I. Levin. The pneumatic part of the micromanipulator enabled one to slit and remove the membrane with smooth and sufficiently precise movements of the needle.

The isolated frog head was placed in saline in a horizontal bath the bottom of which acted as the indifferent electrode.

Histological examination of the saccular branch of the VIII$^{th}$ nerve of the frog was kindly undertaken for us in the Institute of Biological Physics by V. Ye. Shungskaia and T. I. Zelikina. They established that the bodies of the neurones lie in the ramulus itself at about half its length and the site of their location, from external morphological signs, does not differ from the other parts of the ramulus.

The bodies of the cells are of a round-elongated shape and can be quite easily differentiated among the other nerve formations (staining according to Shabadash). They vary in size from 5 to 10 $\mu$.

In order to record the biopotentials from the individual elements of the auditory nerve tract, so-called glass pipette microelectrodes filled with 3 M KCl solution were used as well as the steel needles which were used in most of the experiments.

These electrodes were prepared by means of a simple device built by us, making it possible to reduce the cross-section of the point to a value of less than 1 $\mu$ by passing a current and raising and lowering the steel needle into a corrosive mixture. The input resistance of these electrodes was less than 1 M$\Omega$ and varied with the quality of the covering insulating layer (bakelite or veniflex).

In order to record the biopotentials the cathode follower described by Byzov and Bongard [11] was connected to an OB-2 amplifier and the currents were recorded on a MPO-2 loop oscillograph.

Introduction of the electrode into the body of the cell or fibre was controlled by a d.c. semi-conductor indicator produced in our laboratory.

The sources of sound stimulation were a generator of noise and three audio generators. In a number of experiments the output of the generators was converted into modulated pulses of constant form with a duration of 60 msec by an electronic chopper. The pulses formed were fed to the input of a loudspeaker of uniform characteristic in the frequency range used by us.

The circuit made it possible to obtain alternation of pulses of noise and pure tones with a definite set rhythm (one pulse every 3 sec) and, in addition, to vary the pitch of the tone by any combination of pulses.

The noise pulse was used in order to establish the presence of activity, due to sound stimulations, in the test nerve.

The circuit and the design of the electronic chopper and the noise generator were kindly sent to us by L. A. Varshavskii.

## RESULTS

A total of 60 experiments was made with recording of the potentials from the different parts of the saccular ramulus of the VIII$^{th}$ nerve by stabbing the tip of the electrode into the ramulus. In our experiments the lifetime of the cell or fibre into which the electrode was inserted was very short — a matter of some tens of seconds.

From our observations on the saccular branch of the VIII$^{th}$ nerve there are elements present with spontaneous activity. These do not respond however, to sound stimulation over the frequency range adopted by us. Together with these elements there are also others lacking in spontaneous activity but responding to sound stimulation with well marked action potentials. Many spontaneously active elements were also able to respond to stimulation by a tone or noise.

A sample of recording of spontaneous activity is given in Fig. 2. The spontaneous pulses did not develop rhythmically but with intervals of about 40 msec. In other experiments, somewhat different intervals were observed between the individual spontaneous spikes but we did not observe regular rhythmical spontaneous activity.

The biocurrents were recorded from the neurones as can be judged from the quite large amplitude of the spontaneous potentials — of the order 1 mV. Biphasic currents were recorded.

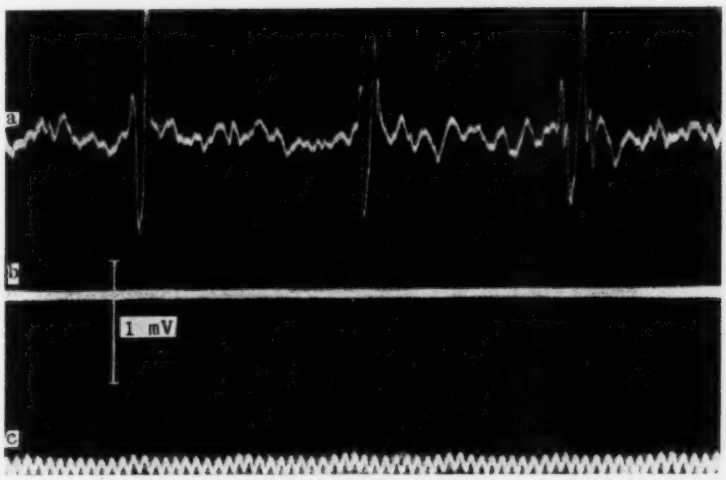


FIG. 2. Spontaneous activity of nerve element of saccular branch of VIII$^{th}$ nerve. (a) record of spontaneous pulses; (b) record of stimulus; (c) time marking.

Examples of the response to the noise pulses are given in Fig. 3. In response to noise pulses with a duration of 60 msec a number of rhythmical spikes formed, the frequency of which varied with the amplitude of the stimulus. A prominent feature was the group of high frequency spikes appearing at the end of the action of the stimulus. Such groups were observed very often in frogs although the nature and origin of these groups of spikes is not understood.

The latent period of excitation was on average 4 msec — given sufficient intensity of noise.

As stated above, the stimulation with a noise pulse was applied in order to demonstrate acoustically active nerve elements of the auditory tract. By switching over from the output of one sound generator to the output of another or a third, we matched the frequency of tone producing the most distinctly marked response in the cell formation responding to the action of white noise.

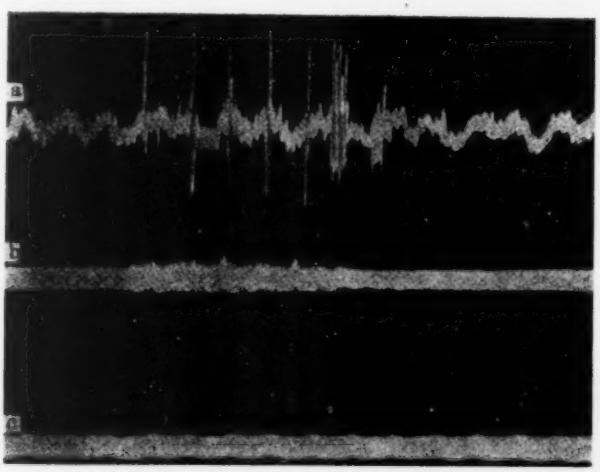

FIG. 3. Action potentials on noise stimulation. (a) record of potentials; (b) record of white noise with intensity of 72 dB; (c) time marking.

An example of the record of the response elicited by the formed pulse of a pure tone is given in Fig. 4.

For a sufficiently formed pulse, a tone with a definite frequency and with an intensity of 76 dB above the level of $2 \times 10^{-4}$ two groups of spikes appeared with a latent period of 5 msec. At the start of the action of the pulse when its intensity was at the threshold level, the nerve element responded with a long spike. When the tone reached its maximum intensity (76 dB) rhythmical potentials of high frequency forming compact bundles appeared (Fig. 4 (a)). All the spikes in the bundle were biphasic.

Where the intensity of the sound was slightly above threshold (Fig. 4 (b)) the frequency of the spikes diminished and only 1–2 spikes were formed. The latent period increased to 12 msec. The shape of the spikes themselves did not change, their amplitude was about 2 mV and not directly related to the intensity of stimulation.

The experiments described above show that the nerve elements of the saccular branch of the VIII$^{th}$ nerve of the frog possess quite marked selectivity in relation to individual tones.

In the record (Fig. 5) of an experiment using a stimulus of a long tone (these experiments having been carried out by us somewhat earlier), the ability of a nerve element to discriminate a tone of definite frequency is also indicated. Moreover, the tone fre-

quency of 730 c/s used in this case is the tone of highest frequency which produces potentials and can be considered as the upper limit of tones to which the nerve formations of the frog auditory apparatus can respond.

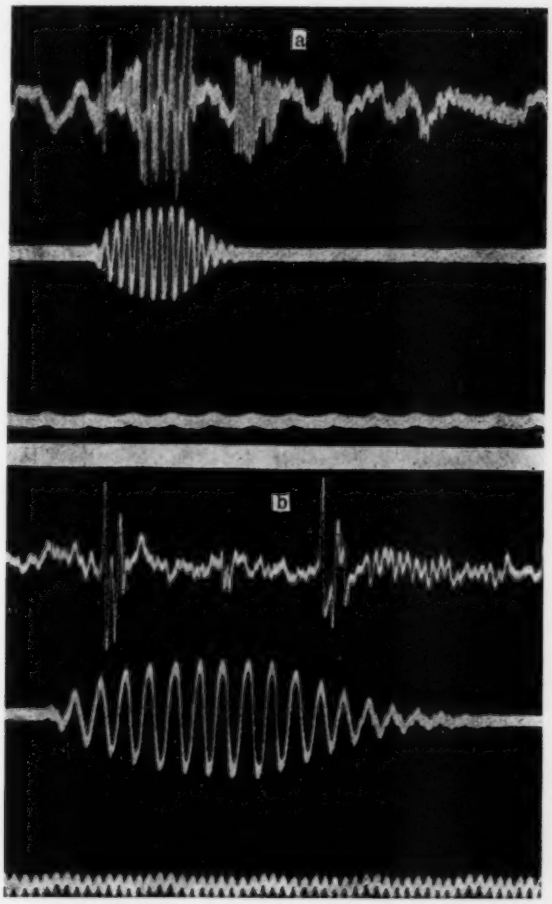


FIG. 4. Action potentials on stimulation with formed pulse (symbols as in previous figure). (a) formed pulse delivered with intensity of 76 dB; (b) formed pulse of near-threshold intensity.

Figure 5 shows the response from the auditory nerve tract obtained from recordings with the glass pipette microelectrodes (with the section of the working lumen within the limits of 1 μ).

The long sounding tonal pulse, i.e. a tone supplied to the loudspeaker directly from the sound generator without use of the electronic chopper gave the responses shown in Fig. 5 (a) and (d). The frequency of the effective tone in Fig. 5 (a) was 730 c/s. The tracings (b) and (c) in the same figure show absence of response to tones of 720 and 740 c/s.

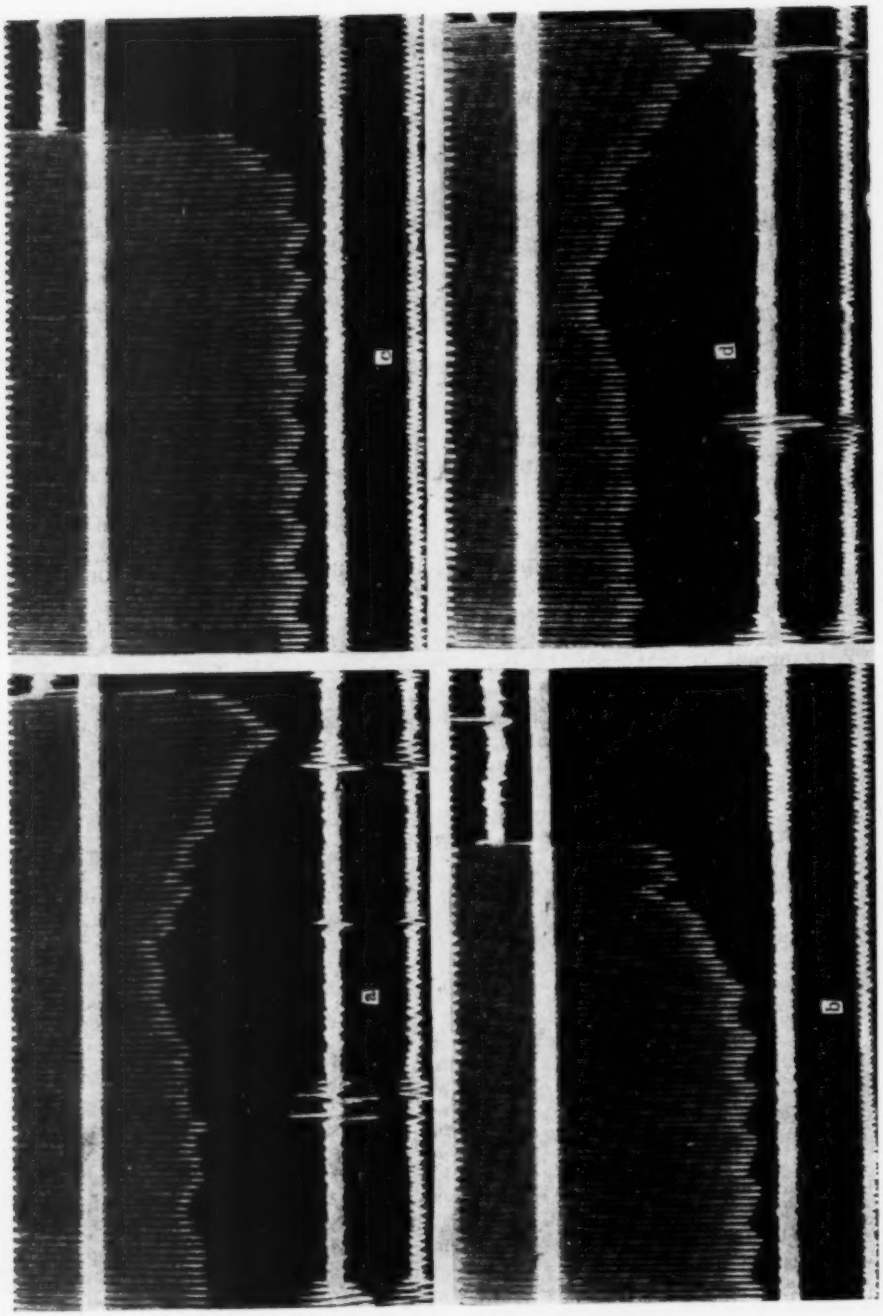


FIG. 5. Action potentials on stimulation with long tone (symbols as before). (a) stimulation with tone of 730 c/s; (b) 720 c/s; (c) 740 c/s; (d) 730 c/s.

VOL
5
196

Record 5 (d) shows that on reversion to a tone of the original frequency (730 c/s) the nerve element again reacted with a group of spikes but of smaller amplitude, this is probably connected with lowering of the vital activity of the element caused by the trauma inflicted by insertion of the electrode.

## DISCUSSION

The findings described in this article still do not allow conclusions to be drawn on the patterns of discrimination of a useful signal in the nerve conducting pathway of an animal with primitive organization of the inner ear (frog). However, even at this stage we can see that the individual nerve elements of the auditory analyser of *R. radibunda* are selective in relation to different sound stimuli.

The primitive structure of the receptor part of the frog ear, as compared with the organ of corti in highly organized animals, apparently does not exclude the possibility of a local electrical response in the nerve elements of the conducting pathway of the organ of hearing of the frog.

A detailed investigation of the significance of the thresholds of the individual elements of the auditory tract of the frog, as has been done for mammals (guinea-pigs, cats, dogs) should be made, as solution of this problem is essential to an understanding of the rôle of the mechanical and nerve elements of the auditory analyser in the system of coding of information.

*Translated by* A. CROZY

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960

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* There is a translation into English of this work.

# ADEQUATOMETRY OF FAST DEVELOPING AUDITORY ADAPTATION (MASKING OF SHORT BY LONG SINUSOIDAL TONES)*

S. N. GOL'DBURT

Ukhtomskii Physiological Institute, Leningrad University

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VOL
5
196

IN a number of earlier papers [1–5] we investigated how the evaluation by man of sound stimulation changes at short time intervals after the termination of the preceding stimulation. These changes are connected with the cycle of recovery of excitability of the nerve or with the interval for which the aural functions are affected after excitation [6, 7], characterized by the degree of change in the threshold.

The interval for which functions of the human auditory (and any other) analyser is affected is extremely complicated; not only does the level of the auditory threshold change but also, the assessment of the loudness and tone of the second sound stimulus closely following the first [3–5]. We have considered these phenomena, adopting the "adequatometric" approach proposed by Makarov [6–8] which presupposes the presence of selective differentiated excitability to the various stimuli. Excitability thus understood is not uniform — it changes in changed functional states, stimulations more suitable for one functional level are less suitable for another.

The "after-shift" in the auditory threshold has also been measured after short intervals of time by a number of foreign workers [9–12] some describing it as "fast developing auditory adaptation" and others "residual masking". The affinity between masking (a persistent rise in the auditory threshold against a background of continuous sound) and fast developing adaptation (the residual rise in the threshold after the sound has just ended) has been demonstrated by Luscher and Zwislocki. However, they noted that the masking cannot be measured for the sound of the masking frequency and that fast developing adaptation is measured not during the actual action of the adapting sound but at a minimum of 10 msec after its termination and it is, therefore, impossible to combine these related phenomena.

If it is agreed that the concept of "masking" (which, of course, we shall continue to use) is essentially descriptive, then we are justified in assigning masking (as a reduction in auditory excitability against a background of continuous sound) to the same class of phenomena as reduction in visual excitability against a background of illumination, i.e. to negative adaptatory shifts†, developing very rapidly. Although the concept

* Biofizika **5**: No. 4, 420–429, 1960.

† Makarov [13] (p. 11) distinguishes positive adaptation (for vision in the dark, for hearing in silence) and negative adaptation (effect of light, sound, heat, cold, etc).

of adaptation is also fairly descriptive, juxtaposition of adaptation and masking in the above mentioned papers, dealing with the very brief after-shift of the auditory threshold, is a step forward.

In order to arrive at an explanation of the link between adaptation, masking and general physiological concepts on the fall in excitability, we measured the thresholds of short pure tones against a background of relatively long pure tones, since in the literature we have been unable to find such information; we are only familiar with work on masking of short tones by noises [14–16] and masking of clicks by pure tones [17]. We sought first to measure the adaptation and masking simultaneously and secondly, to discover different types of shift in the threshold of masking for tones of different durations, since the masking tones of different frequencies and intensities alter the state of the auditory analyser in various ways by shifting its level of adaptation and may exert a different influence on the thresholds of the test masked tones of different durations. It seems to us that the experimental results correspond to those suggested by the theory of adequatometry.

VOL.
5
1960

## METHODS

The threshold short test tone (masked sound or signal) and the adapting background tone (masking sound or interference) were supplied from two ZC 10 audio generators to the same TD-6 electrodynamic headphone and were heard by the subject monaurally in a sound-proof room. To avoid the reciprocal effect of both circuits on the circuit of the ZG-10 audio generator supplying short tones an additional resistance was introduced. The headphones were calibrated against "the artificial ear" kindly made available to us by I. K. Ioffe. The non-uniformity of the frequency characteristic from 100 to 5000 c/s did not exceed $\pm 6{\cdot}5$ dB. The frequency indicators of both ZG-10 audio generators were calibrated with a 50 c/s standard, by the method of Lissajous figures on an EO-7 oscillograph; the Lissajous figures were simple thanks to the simultaneous use of two audio generators. The short tones were mechanically timed by means of a pendulum contact along the stand of which slid a small brush of flexible springy strings [18]. The duration of the masked sound was determined by the time of the brush sliding along the stand contact. The contact was connected to one terminal of the headphone. With the terminal disconnected, a sufficiently strong tone continues to be faintly heard by the listener because of the spurious capacitance, but by careful shielding of the lead from the audio generator to the contact, this complication can be eliminated. The durations of the short tones used were at first measured mechanically with a tuning fork; checking of the measurement on the ENO-1 oscillograph introduced a slight correction. The steepness of the rise and fall of the short tones, as indicated by the ENO-1 oscillograph did not exceed 10 μsec.

Four frequencies of the masking tone — 200, 500, 990 and 5000 c/s and four frequencies of the masked tone — 200, 500, 1000 and 5500 c/s were investigated.

A systematic investigation was made of three durations of the masked tone: 5 msec, 22 msec and 66 msec — and six graduations of intensity of the masking tone from the threshold of sensation up to 90–100–110 dB above the threshold (the curves of masking

at 10 dB above the threshold are not given in Fig. 1). Sometimes a masked tone with a duration of 1 msec was used.

Five subjects with normal hearing took part in the experiments. They were first trained for 2–3 weeks to hear threshold short sounds in silence i.e. in the absence of a masking tone; the first two experimental measurements of the thresholds against a background of strong sounds were discarded. The threshold was always measured by progressing from an as yet, inaudible level of sound to an audible one. The task of the subject was to detect a short sound against the background of a long one. The subject did not report on the long sounds. The thresholds of the short tones in silence were repeatedly checked. A total of 20 experiments were performed on each subject and for each of the 288 variants of the conditions not less than five different measurements were made. In a given experiment the threshold was considered to be reached if three positive responses were obtained in succession. The duration of the masking tone was varied from 0·5 sec to 5–10 sec, rarely up to 30–60 sec since a special preliminary check showed that the shift in the threshold of the short sound for these durations was uniform. The threshold of the masking tone was also constant during 30 min of its action [19, 20].

VOL
5
196

## RESULTS OF EXPERIMENTS

The results obtained, as Fig. 1 (A–D) shows, strongly resemble the typical masking curves of Wegel and Lane [21], which might have been expected on the basis of the physics of short tones [22]. In fact, the shorter the sound the greater its spectrum deviates from a pure tone. The frequency band width of a short sound is $2/t$; the width of the effective band 1/t where $t$ is the duration of the sound (in sec). Thus, for $t = 5$ msec the width of the effective band will be $1/5 \times 10^{-3} = 200$ c/s. This means that a tone of 200 c/s lasting only 5 msec is converted into a complex sound with a frequency band from 100 to 300 c/s with a maximum at 200 c/s, i.e. is converted from a pure tone into noise. In fact, this sound is heard as a click not like the fairly long tone of the same frequency. A tone of 5500 c/s for $t = 5$ msec is transformed into a sound with a frequency band of from 5600 to 5400 c/s, i.e. changes comparatively little and consequently, we hear it as a very minute bell, more accurately the element of the bell, still resembling however, a long tone of the same frequency. A tone of 200 c/s for 66 msec is converted into a sound with an effective frequency band width of $\sim 15$ c/s, i.e. from 192 to 207 c/s and, consequently, will differ little from a long pure tone and tones of higher frequency of this duration will differ from pure tones even less.

The shift in the threshold against a background of a long strong sound (upper curves in Fig. 1) is characteristically asymmetrical with a sharp drop towards frequencies lower than the background and a stable level at high frequencies.

Where the frequencies of both tones coincide the short tone for 66 msec which in silence fully resembles the long pure tone, will, against a background of the masking tone of sufficient intensity (30–40 dB above threshold) sound like a click. For it to become audible in its individual tonality, it must be amplified. Measurement of the degree of masking from the threshold of appearance of tonality in a short tone against

a background of a long one of the same frequency was not our main task; however, this change is of great importance. Fig. 2 shows the divergencies of the tonal and atonal (click response) of the thresholds of the masked tone of 200 c/s and 1000 c/s with $t = 66$ msec. These are the mean results of a few experiments on the same subject. They resemble the results of Chistovich [23]; (1) for an intensity of 55 dB of the masking tone of 990 c/s, the atonal threshold of the masked tone is equal to 30 dB, the tonal threshold to 55–52 dB; and (2) the masking curve falls steeply towards lower tones. For 200 c/s the difference in the atonal and tonal thresholds in relation to the intensity of stimulus varied from 2 to 10 dB, instead of 6–20 dB for 1000 c/s.

In our view the following observation is important: when a tone for 66 msec becomes audible as the pure tone but against a background of a tone of close or coincident frequency this does not mean that both tones are audible at the same time; at that instant when the short tone is audible the loud masking tone as it were, vanishes.

VOL. 5 1960

At the level of perception of the masking tone of 990 c/s, 100 dB, the short tone of 1000 c/s, 66 msec, 90 dB is heard as a click over the 940–1040 c/s range; at the boundaries of this zone a click is no longer heard but a lower tone or correspondingly a tone higher than 1000 c/s is heard. Perhaps later, by studying this phenomenon in detail, it will be possible to use it in the same way as the method of beats for sounding of subjective overtones.

The curves in Fig. 1 do not show the troughs formed by the beats of the masked tone for close frequencies of the masking and overtones. We believe that the method of masking of a sufficiently pure short tone (60–100 msec) by a long tone may be of practical value in a number of cases. Reproduction of the curves of masking of short test tones opens up new possibilities for investigation in a simple and methodologically convenient form. A short tone (5 msec; 1 msec) was detected against a background of a long masking tone even more readily than in silence (this has been noted for short tones masked by noise). At the same time, methodologically this procedure of masking of a tone by a tone is simple compared with, for example, the interesting, but very complicated procedure of masking a tone with a combination of tones [23] which is practicable only in a very well equipped laboratory. Our experiment can be performed by the otiatric physician and clinically such an analysis of masking undoubtedly promises interesting findings.

Further, differences were detected in the degree of rise of the threshold (taking the threshold in silence as starting point) for tones of different durations. However, these differences clearly apparent in Fig. 1, were not always statistically very high but with definite frequency ratios the differences were quite significant (the reliability of the difference was determined by Student's method, see Fisher [24]). Thus, if the frequencies of the masking and masked tones were close or coincided, the lowest thresholds for all the frequencies were obtained for the shorter durations of the masked tone, i.e. for 5 or 22 msec. The significance of the minimum value in the shift in the threshold for these durations, as Table 1 shows, was not adequate for all the gradations of intensity of the masking tone; the probability of the "zero-hypothesis" for certain intensities lay between 0·1 and 0·5 but for many intensities it was adequately high.

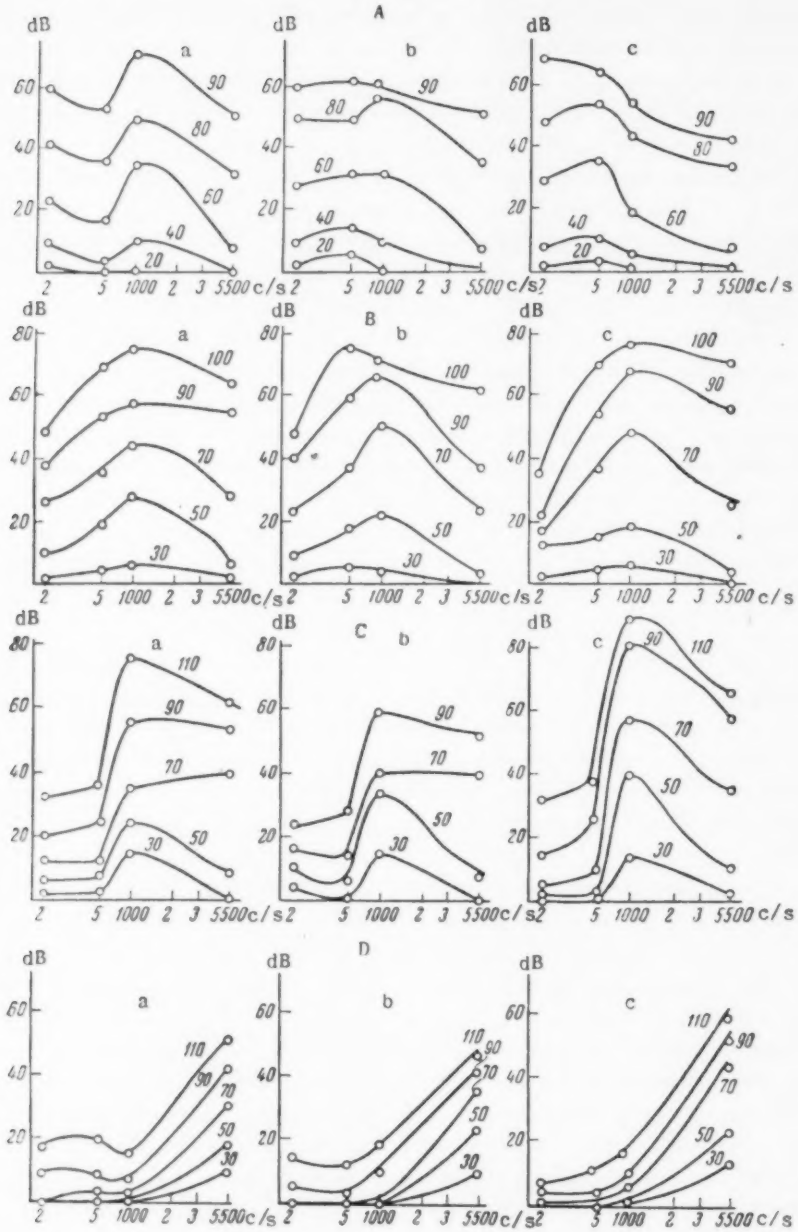


FIG. 1. Masking curves of short sinusoidal tones by a long one. Frequency of masking tone for A — 200 c/s; B — 500 c/s; C — 990 c/s; D — 5000 c/s. (a) duration of short masked tone 5 msec; (b) 22 msec; (c) 66 msec. Abscissa, frequency of masked tone; ordinates, increase in its threshold in dB (threshold in silence taken as zero). Numerals above curves indicate level of masking tone in dB above threshold of sensation (mean data for five subjects).

Tables 1 and 2 give only the findings for which the probability of the zero hypothesis is equal to 0·01 or 0·05, i.e. the significance of the difference is very high. The tendency noted for other intensities was thereby validated (see Fig. 1, A–D). A minimal shift

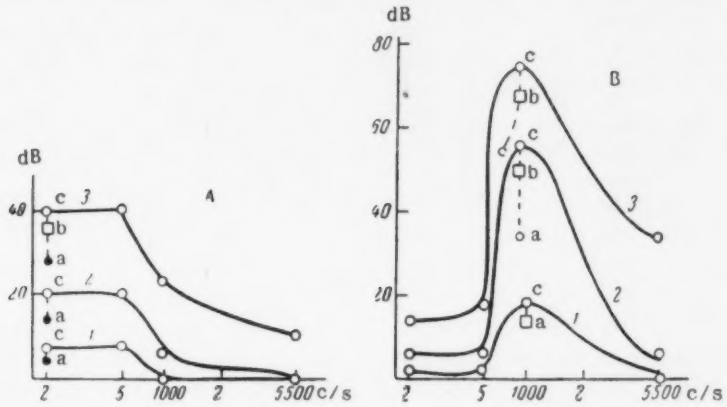


FIG. 2. Degree of masking of short tone of 66 msec duration by a long tone in relation to criterion of tonality of threshold. A, masking frequency 200 c/s; B, masking frequency 900 c/s. Level of masking tone for curves 1, 2 and 3 of graph A 20, 40 and 60 dB respectively; 20, 55 and 75 dB for curves 1, 2 and 3 of graph B. (a) "click" response; (b) "disturbed tone" response; (c) "tone" response. Mean data for subject S.G.

in the threshold (on correspondence or closeness of the masked and masking frequencies) was demonstrated for shorter masked tones of 5 or 22 msec duration; the curve for 66 msec passes, as a rule, considerably higher (see Fig. 1). As Table 1 shows, the high significance of the difference suggesting easier detection of short tones, was

TABLE 1. INTENSITY LEVELS OF MASKING TONE FOR WHICH A SIGNIFICANTLY SMALLER SHIFT IN THRESHOLD FOR SHORTER TONES (5 OR 22 MSEC) THAN FOR A LONGER TONE (66 MSEC) WAS DEMONSTRATED

| Masking frequency (c/s) | Masked frequency (c/s) | Intensity level of masking tone above threshold (dB) |
|---|---|---|
| 200 | 200 | 60; 80 |
| 200 | 500 | 20; 40; 60; 80; 90 |
| 500 | 20 | 50 |
| 500 | 500 | 10; 100 |
| 500 | 1000 | 70; 90; 100 |
| 500 | 5500 | 90; 100 |
| 990 | 500 | 90 |
| 990 | 1000 | 50; 70; 90; 100 |
| 990 | 5500 | 30; 90; 100 |
| 5000 | 1000 | 70 |
| 5000 | 5500 | 10; 50; 70; 90; 100 |

TABLE 2. INTENSITY LEVELS OF MASKING TONE FOR WHICH A SIGNIFICANTLY LARGER SHIFT IN THRESHOLD FOR SHORTER TONES (5 OR 22 MSEC) THAN FOR A LONGER TONE (66 MSEC) WAS DEMONSTRATED

| Masking frequency (c/s) | Masked frequency (c/s) | Intensity level of masking tone above threshold (dB) |
|---|---|---|
| 200 | 1000 | 40; 60; 80; 90; 100 |
| 500 | 200 | 70; 90; 100 |
| 500 | 1000 | 10; 50 |
| 990 | 200 | 50; 70; 90 |
| 990 | 1000 | 10 |
| 5000 | 200 | 70; 90; 100; 110 |
| 5000 | 1000 | 90 |

demonstrated at a coincident frequency of 200 c/s only two levels — 60 and 80 dB but the same tendency was also clear for the other levels of masking; for almost coincident frequencies of 990 and 1000 c/s and close frequencies of 5000 and 5500 c/s the

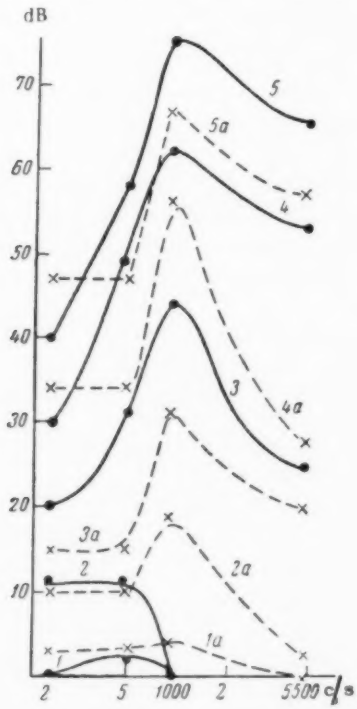


FIG. 3. Masking curves of tones of 22 msec duration (curves 1–5) and tones of length 1 msec (curves 1a–5a) by tone of frequency 500 c/s. Level of masking tone for curves 1 and 1a — 20 dB above threshold; for 2 and 2a — 40 dB; for 3 and 3a — 60 dB; for 4 and 4a — 80 dB; for 5 and 5a — 100 dB. (Individual experiment with subject T.K.).

significance of the difference was demonstrated quite convincingly — for four and five levels respectively of the six employed. The findings for 500 c/s are the least clear; a significant difference was demonstrated only for very weak (10 dB) and very strong (100 dB) levels of masking but against a background of 200 c/s, shorter tones of 500 c/s were significantly more easy to detect for five of the six levels investigated.

As the frequency difference between the tones increased an inverse relation was observed (or a tendency in that direction) as shown by Fig. 1 and Table 2. A significantly smaller shift in the threshold was obtained for the maximum duration employed. This is demonstrated by Table 2; against a background of 200 c/s a longer (66 msec) tone of 1000 c/s was significantly easier to detect than the shorter ones for five levels of masking of the six used, against a background of 990 c/s and 5000 c/s a tone of 200 c/s for 66 msec was easier to detect for the third and fifth levels. However, comparing Tables 1 and 2 we note, firstly, that for weak levels of masking, easier detection of a longer tone is possible even for coincident frequencies and, secondly, that for a shorter tone the reliability of detection was demonstrated in a larger number of cases than for the longer one. For a still shorter masked tone these relations, checked so far by only a small number of experiments were even more striking. This is shown in Fig. 3 for a duration of 1 msec. As in Fig. 1, the typical picture of masking is seen, but the stronger the masking tone, the weaker its effect on the shortest, i.e. with minimal energy, for tones of the same frequency. This is in accord with the findings in paper [17] on the inability of pure tones to mask very short clicks.

OL.
5
960

## DISCUSSION

The findings presented support the main contention of Makarov's theory of adequate differential excitability: the value of a definite duration of the masked tone is not absolute and depends on which sound stimulations are involved in the interaction — those of similar or different frequencies. For close or coincident frequencies of both tones the smallest shift in the threshold was given by a short tone of 5 msec, heard as a click and therefore contrasting distinctly with the masking tone which more easily suppressed the perception of an almost identical tone of duration of 66 msec. Conversely, against a background tone of 5000 c/s sufficient frequency contrast was displayed by tones of 200 and 500 c/s with a duration of 66 msec. There was a smaller rise in threshold and the tones were more readily detectable than for durations of 5 or 22 msec with 3 and 13 times less energy. In this case, the "noise" contrast of shorter tones was not high and did not predominate over the energy factor. Similar phenomena have been obtained on masking of short tones with noises [15] and clicks with pure tones [17]. The shorter the tone, the more does noise raise the threshold since the short tone is similar to noise and is not picked out in such "relief" [15] against a background of noise as is the longer and purer tone. A pure tone is almost unable to mask short (lasting several microseconds) clicks with their vast noise-like spectrum. It is possible that a short tone (5 or 22 msec) does not act on the auditory analyser only as the sum of its frequency components. Its reflexion is probably not confined to peripheral frequency analysis but causes in the nerve fibres and centres a peculiar form of signalling

which A. A. Ukhtomskii has described as an "ensemble of excitations" and American workers as "pattern of excitation". The entirety and indivisibility of the total pattern of signalling can hardly be reflected solely by peripheral processes, however important they may be, and is probably due to the participation of the centres.

Our observations compel us to think again on what constitutes masking.

In one of his books [25] Makarov notes that separation of general physiology and the physiology of the analysers has led to the description of an unjustifiably large number of diverse phenomena for each sense organ. Indeed, only quite recently [11] did the similarity of masking and adaptation become obvious but both concepts are descriptive and do not characterize the inner meaning of these phenomena. In the general physiology of excitation a subject of unabated controversy for more than half a century has been the nature of the refractory-nonexcitability interval during which the excited tissue does not respond to any stimuli. Physiologists studying hearing have measured masking and negative adaptation, i.e. the manifestation of refractoriness, since masking is nothing other than reduction in excitability of those elements of the auditory system engaged at a given moment in responding to stimulation. Auditory masking and refractoriness of excitable tissues could be described as expressions of the incompatability of two or more full-size excitations in the same excitable elements. Makarov has repeatedly [26] stressed that excitability and the competence of living tissue are not the same thing and this is confirmed by close consideration of masking and refractoriness. The nerve engaged in the transmission of excitation is competent to the highest degree, it is at the apogee of the active state and precisely because of this it is non-excitable since additional excitation would be biological interference. Likewise, masking cannot be defined as the incapacity for analysis [27] if we mean by analysis resulution of a complex whole into diverse components. Masking is first and foremost a state of strong excitation naturally accompanied by "covering" of homogenous weaker excitations [21]; frequencies differing from the masking one are "covered" only by spread of excitation along the basilar membrane, i.e. also by analogy with excitation, dominating at a given time the auditory analyser. Consequently, masking in hearing, in the same way as refractoriness for the nerve, is the inability to respond because the responding instrument is already engaged.

The same elements are incapable of responding simultaneously to two stimulations. When a short sound (66 msec) reaches its tonal threshold against a background of a long sound of the same frequency (Fig. 2) this does not mean that one can simultaneously hear both sounds. At the instant when the short sound is heard the long sound is not heard and it, as it were, vanishes. It is impossible to hear two sounds simultaneously and the masking sound is now the short now the long sound; setting the signal against noise, thus becomes relative.

Negative adaptation is very similar to masking if it is regarded as a subsequent or concurrent stimulating rise in threshold. But the concept of adaptation is wider for it characterizes not only the fall in excitability, but a certain drop in competence (lessening of loudness of the sounding tone).

VOL
5
196

Of course, the outward similarity of masking and refractoriness still does not prove them to be of a common nature since the dimensions of the intervals differ: the after-shift in the auditory threshold, i.e. the residual masking can last for hundreds of milliseconds. But as we cannot rule out the participation of the central sections of the auditory analyser in these processes, extension of the intervals ceases to be an obstacle to working analogies and as yet, we cannot lay claim to anything more than this.

## SUMMARY

(1) The curves of the adaptatory shift in the threshold of short tones of 5, 22 and 66 msec duration and frequency 200, 500, 1000, 5500 c/s against a background of long acting tones of different intensities and frequencies (200, 500, 990 and 5000 c/s) closely resemble the masking curves of Wegel and Lane. Consequently, it is easy and simple to measure the degree of masking, of a tone by a tone, using short masked tones which are more distinctly discernible against a background of a long tone than is a long tone against a background of another long tone.

(2) The results open up the possibility of measuring the threshold of tonal masking with coincident or close frequencies of the masking and masked tones using a short ($\sim$ 100 msec) and at the same time sufficiently pure tone and measuring its thresholds of detection from atonal ("click") to tonal.

(3) If the frequencies of the masking and masked tones coincide or are close then the threshold of the shorter masked tones increases less than that of longer tones.

(4) The further apart the frequencies of both tones the more significant and reliable the increase in the threshold for shorter durations of the masked tone as compared with the longer one.

(5) The findings described extend the theory of adequate selective excitability developed by Makarov and suggest an affinity between masking and rapidly developing auditory adaptation and the phenomenon of reduction of excitability against a background of the active state, i.e. with the refractory period of the interval of functional shifts.

*Translated by* A. CROZY

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VOL
5
196

# ANALYSIS OF THE KINEMATICS OF THE HUMAN BODY ON CARDIAC CONTRACTION (VECTORBALLISTOCARDIOGRAPHIC INVESTIGATION)*

V. L. KARPMAN

Institute of Normal and Pathological Physiology, U.S.S.R. Academy of Medical Sciences, Moscow

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As far back as 1877 Gordon [1] attempted to record the mechanical processes developing as a result of cardiac activity by recording the movements of the human body. Later, a number of workers applied this principle and, with the development of the technique of measuring the mechanical magnitudes, improved on it. Thus, already in 1933, Abramson [2] designed for this purpose a special device with a natural frequency of vibration of 75 c/s. However, recording of the vibrations of the body came into wide use only following the work of Starr [3] and, later, Nickerson [4] who designed ballistocardiographic instruments with a sufficiently high quality of reproduction of

* Biofizika **5:** No. 4, 430–437, 1960.

the shifts in the body from cardiac contraction and movement of the blood along the vessels.

At the beginning of this century, Henderson [5] remarked that the shifts in the human body are orientated not only in a longitudinal, but also in a transverse direction. Systematic investigation of the shifts in the human body in two and three-dimensional co-ordinates was initiated in 1945 following the work of Hamilton [6]. Soon, special instruments were designed — vectorballistocardiographs (see [7]) to study the displacement of the human body in a given plane.

Vectorballistocardiographs usually consist of two amplifying channels, the voltages from the output stages of which are supplied to the opposite plates of a cathode ray tube. If from one such channel an electric signal is delivered characterizing the shifts in the body about axis X (Fig. 1) and to the other about axis Y, then a curve will be obtained on the cathode ray tube — the vectorballistocardiogram — representing the displacements in the human body in the XY plane. Likewise, vectorballistocardiograms can be obtained for the XZ and YZ planes.

OL.
5
960

Together with this automatic procedure for constructing the vectorballistocardiogram the curve for the displacement in space of the human body can be obtained by normal geometric techniques. At the points characterizing the shifts in the body in the three mutually perpendicular directions there are the points of the vectorballistocardiograms corresponding to given moments of time.

The latter method is not suitable for practical purposes since it is very laborious, but in theoretical investigations it is irreplaceable allowing easy correlation of the character of the curve under study with the physiological processes occurring in the cardio-vascular system.

The vectorballistocardiogram is a curve characterizing the size and direction of the displacements of the human body at different moments of the cardiac cycle relative to the nominal "position of rest". It is understandable that such a "position of rest" can only apply if no forces are acting on the body and, in particular, no stresses produced by the work of the blood circulatory apparatus. Since such a requirement is not applicable to the living body we shall call the "position of rest" nominal.

The pattern of the vectorballistocardiogram is determined not only by physiological processes but depends on the natural frequency characteristics of the instruments recording the displacements. From the data in the literature known to us, most vectorballistocardiographic investigations have been done on the high frequency table of Starr [6–8]. A low frequency table with critical damping was used by Franzblau [9]. The preference for high frequency tables, it seems to us, is not dictated by any fundamental advantages over low frequency ones and is probably due to the wider use of instruments of this type. An attempt to use the instruments of Dock [10–12] for vectorballistocardiography is not theoretically justified as it is known (Kazmeier and Schild [13]) that displacements in the various parts of the body do not occur in phase.

Vectorballistocardiography was previously applied both in clinical and theoretical investigations. It was thought that by examining patients with this technique it would be possible to find characteristic changes in the curve which could be used for diagnostic

purposes in various heart conditions. However, these attempts have so far been unsuccessful in pathological conditions, although the vectorballistocardiogram changes its pattern (Brandt [10]; Braunstein [7]; Dock [14]; Morris [15]; Legrand [12], etc.) it does not present pathognomonic pictures.

## METHODS

In order to construct vectorballistocardiograms, an analysis was made of the curves recorded on the special high frequency table designed in the Laboratory of Clinical Physiology of the Institute of Normal and Pathological Physiology of the Academy of Medical Sciences of the U.S.S.R. in conjunction with the Research Institute for Weights and Instruments. The table consists of a platform connected to a massive base by a system of beams on which are sealed wire strain gauges. The subject is placed on the platform and the vibrations of the body produce deformation of the beams and change the resistance of the transmitters. As the latter are connected to a bridge circuit, acting as the arms of the bridge, a change in resistance is characterized by the appearance of an electric signal which is amplified and then either recorded on photographic paper by a loop oscillograph or fed to a cathode ray tube. This instrument permits synchronous recording of the displacements orientated along the three axes of co-ordinates (Fig. 1).

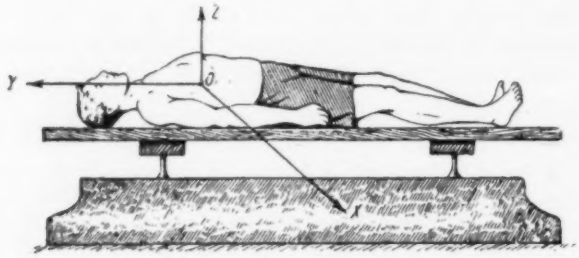


FIG. 1. Arrangement for recording displacements of body. YOX, frontal plane; YOZ, saggital plane; XOZ, transverse plane.

In the present work synchronous recording was made of the shifts in the human body about axis Y, i.e. in the head to foot direction (longitudinal ballistocardiogram), about axis X (the so-called lateral ballistocardiogram) and about axis Z in the thoraic-dorsal direction for a subject in a reclining position (dorso-ventral ballistocardiogram). These three kinematic processes were recorded on a loop oscillograph with 120 mm photographic paper. In order to construct a qualitative time curve of the displacements of the human body a high scanning speed of 125 mm per sec was used and the time marking was 0·01 sec.

The time curve was plotted from points, the co-ordinates of which were determined from the value of the deviation of each of the three ballistocardiograms from their zero line. The co-ordinates were determined every 0·03 sec.

Before the investigation proper, the instrument was loaded with weights of 200 g in all three directions so that the deviation of the beam on the photographic paper of the oscillograph was 20 mm.

## RESULTS OF INVESTIGATION

The vectorballistocardiogram was constructed from the points lying within the *G–N* interval of the longitudinal ballistocardiogram, the later diastolic waves P, O, etc were not analysed because of their inconstancy and, with a comparatively high cardiac rhythm, frequent absence.

The greatest amplitude in the displacements of the normal human body was recorded on the longitudinal ballistocardiogram while the maximum amplitudes for the lateral and dorso-ventral ones were considerably less in normal conditions. If the amplitude of the displacements about axis Y is taken as 100 per cent, then the ratios of the amplitudes along all three axes were roughly Y : X : Z = 100 : 38 : 15. Similar results were obtained by Dock for normal conditions [14].

We shall in the first place deal with a description of the vectorballistocardiogram in the frontal plane (XOY in Fig. 1). As shown by Fig. 2 this curve is of complex configuration. Unlike the ballistocardiogram, the course of the vector curve of the shifts differs fundamentally for different subjects. However, by making a permissible simplification and analysing only that part of the curve describing the displacements during cardiac contraction, we can distinguish three morphological variants of the curve. The first variant covers curves with a positive direction of rotation, i.e. clockwise arrow; the second, those with a negative direction of rotation; and the third represented by curves of mixed direction of movement (Fig. 2 (A), (B) and (C)).

In contrast to the inconstancy of the pattern, the disposition of the main points of the vectorballistocardiogram with respect to the axis of co-ordinates was comparatively constant for the different subjects examined. We describe as the main points of the time curve of the shift in the body those which correspond in time to the points of the longitudinal ballistocardiogram, they are given the same symbols as for the longitudinal curve with addition of the subscript v.

The radius-vector $R_H$ drawn from the origin of the co-ordinates to point $H_v$ forms together with the positive direction of the Y axis an angle equal on average to 14° with a maximum deviation of $\pm 10°$; the radius-vector $R_I$ an angle of $259 \pm 25°$; the radius-vector $R_J$ $85 \pm 10°$; $R_K$ an angle of $287 \pm 20°$. Similar data were obtained by Braunstein [7], March [11] and Morrison [15].

The relationship between the variation of the trajectory of the displacement of the human body and different physiological factors has still not received sufficient study. Individual investigators have shown a relation between the pattern of the vectorballistocardiogram and the position of the heart in the thorax of the subject [16] and the age of the subjects [11]. As already stated, for certain diseases of the blood circulatory apparatus the pattern of the vector curve also changes. We observed changed patterns in patients with aortic coarctation, patent ductus arteriosus and mitral stenosis (Fig. 2 (D), (E) and (F)).

The time curve of the displacements in the human body in the saggital (YOZ) and transverse (XOZ) planes has an even less constant pattern than for the frontal plane The averaged vectorballistocardiograms in these planes are given in Fig. 3.

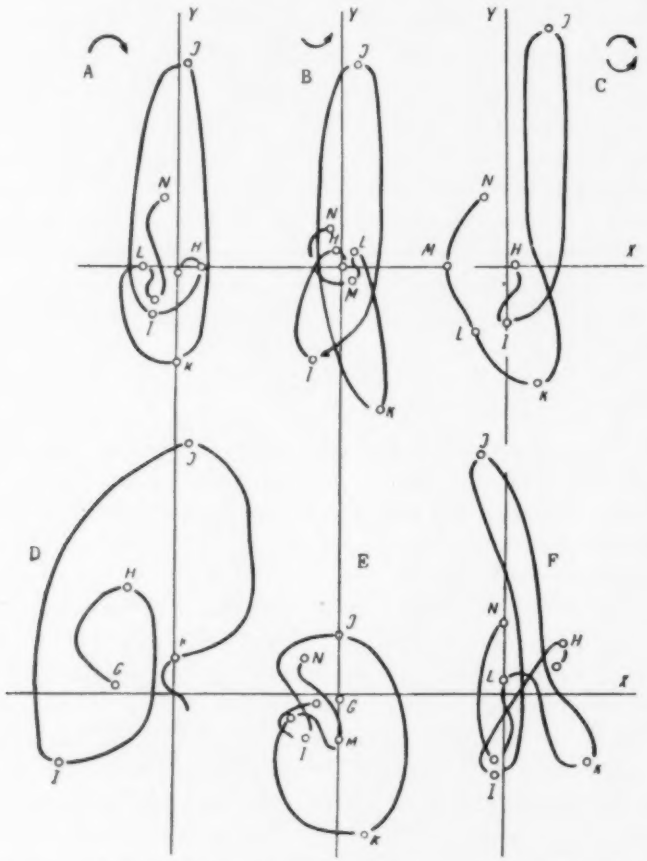


FIG. 2. Vectorballistocardiograms in frontal plane. (A, B, C) normal subjects; (D) vectorballistocardiogram of patient with aortic coarctation; (E) curve for patient with patent ductus arteriosus; (F) curve for patient with mitral stenosis.

The pattern of the three-dimensional vectorballistocardiogram is extremely complex. We therefore attempted to apply certain quantitative indices to define this curve.

The radius-vectors drawn from the centre of the co-ordinates to the points of the time curve may serve as a guide to the size of the shifts in the body with respect to a nominal "position of rest". Since such a radius-vector is in our case a vector function of time

$$\bar{R} = f(t)$$

it is possible to speak of the magnitude of these displacements arising in different phases of the cardiac cycle.

The actual displacements of the body exceed those recorded by the ballistocardiograph since the reaction between the human body and the platform of the instrument on which it rests is far less than the inertia of the mechanical system (Fig. 4). The lack of precision in the ballistocardiographic recording of the displacements is heightened

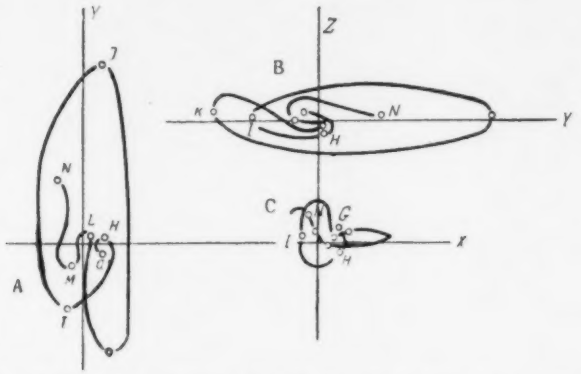


FIG. 3. Vectoballistocardiograms of normal subject in frontal (A), saggital (B) and transverse (C) planes.

in each case by the fact that the links between the body and platform depend on such variables as the weight of the subject, the state of his subcutaneous-fatty layer, the clothes worn, etc.

The modulus of the radius-vectors is computed from the formula

$$R = \sqrt{R_X^2 + R_Y^2 + R_Z^2}$$

(where $R_X$, $R_Y$ and $R_Z$, the projections of the radius-vector on the axis of co-ordinates) and is expressed in relative units. Choice of the values characterizing the displacements of the body from the high frequency ballistocardiogram is somewhat difficult as the instrument records, as stated above, not so much the displacements of the body itself, but rather the displacements of the ballistocardiographic platform induced by the body. The absolute value of the vibrations of the platform relative to the base of the table, for the same force acting on it, depends on the rigidity of construction of the instrument and is therefore not comparable for ballistocardiographs of different systems. The displacement values given in Table 1 were obtained by multiplying the value of the deviation of the beam of the oscillograph by a conversion factor.

TABLE 1. MODULUS OF RADIUS-VECTORS DRAWN TO MAIN POINTS OF THREE-DIMENSIONAL VECTORBALLISTOCARDIOGRAM (ARBITRARY UNITS)

| Category of subject | Radius-vectors | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $R_G$ | $R_H$ | $R_I$ | $R_J$ | $R_K$ | $R_L$ | $R_M$ | $R_N$ |
| Normal | 31 | 27 | 73 | 188 | 118 | 11 | 52 | 71 |
| Mitral stenosis | 10 | 79 | 81 | 212 | 99 | 14 | 61 | 81 |
| Aortic coarctation | 65 | 130 | 122 | 226 | 49 | — | — | — |

As Table 1 shows, the value of the displacement of the body with respect to the nominal "position of rest" varies for different stages of the cardiac cycle — it reaches maximum values during ejection of blood from the heart ($R_Z$, $R_K$) and falls sharply at the start of diastole. In the middle of diastole the displacements again increase followed by a further fall in presystole.

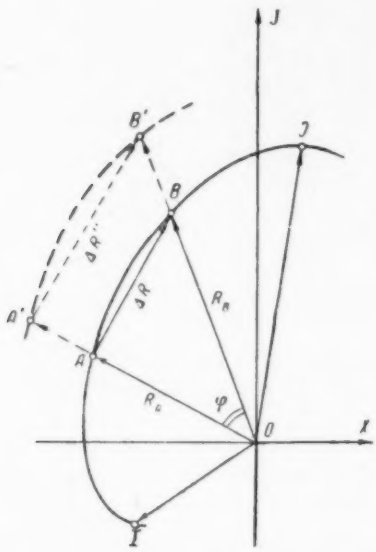


FIG. 4. Time curves of displacement of body ($A' - B'$), and platform of the high frequency ballistocardiograph ($I - A - B - J$). Explanation in text.

In mitral stenosis, the values for the displacement of the body were close to normal. The sole difference was an increase in the displacement at moment $H_v$, obviously related to intensification of the cardiac impulse. In aortic coarctation the displacements of the body differed in considerably size from the normal. They were appreciably increased at the start of ventricular systole and, conversely, reduced at its end. The increase in the displacements is most likely associated with marked hypertrophy of the myocardium and the increased resistance to flow of the blood in the ascending aorta; the decrease in displacements at moment $K_v$ is probably due to the presence of obstruction from which the pulse beat is reflected with reduced energy.

Since the vectorballistocardiogram characterizes a fully explicit physical concept — displacement of the human body in space — analysis of it may be supplemented by a derivative of displacement, e.g. velocity. If the equation for the time curve of the body displacements written in analytical form $\overline{R} = f(t)$ could be expressed in specific form then the velocity of the displacements would be a vector derivative of the radius-vector with respect to time. However, deriving equations of motion for the human body is a complex mathematical task which, apart from anything else, is complicated by the extraordinary polymorphism of the patterns of the vectorballistocardiograms

for different subjects. Therefore, we simplified the problem of differentiation and determined the mean velocity of the displacements of the body for the different intervals of the vectorballistocardiogram, confining the calculation to the main points.

The mean velocity of the displacement of the body $v_{mean}$ was calculated as the ratio of the increment of the radius-vector $\Delta R$ to the corresponding time increment $\Delta t$:

$$v_{mean} = \frac{\Delta R}{\Delta t} = \frac{\sqrt{(R_X - R'_X)^2 + (R_Y - R'_Y)^2 + (R_Z - R'_Z)^2}}{\Delta t}$$

It is obvious that the calculation of $v_{mean}$ must be made in relative units, as in the case of the analysis of the displacements.

Table 2 gives the values obtained by us for the linear velocity of displacement.

These data show that the linear velocity of displacement of the body in space is a variable and that its maximum value was reached in the $I_v - J_v - K_v$ intervals, i.e. during ejection of blood from the heart into the great vessels.

Analysis of the linear velocity of displacement in different subjects revealed considerable individual variations, in a number of cases reaching as high as $\pm 50$ per cent relative to the mean values. It is understandable that this considerably reduces the value of the index and calls for a cautious approach in interpreting it (as we took in discussing the value of the displacement). In fact, the linear velocity of displacement probably exceeds that which may be calculated on the data from a high frequency ballistocardiographic table. It follows from Fig. 4 that if the actual displacements of the body $\Delta R''$ in any time interval do not equal the shift in the platform of the table $\Delta R$, then the linear velocity is not uniform. In order to exclude the error inevitably arising in work with a high frequency table, we decided to calculate the angular velocity of the body displacements.

The angular velocity $\omega$ was determined as a ratio of the angle $\varphi$ through which the radius-vector turns (Fig. 4) to the corresponding time interval $\Delta t$:

$$\omega = \frac{\varphi}{\Delta t}$$

where the angle can be calculated and expressed in radians from the formula

$$\cos \varphi = \frac{R_X \cdot R'_X + R_Y \cdot R'_Y + R_Z \cdot R'_Z}{\sqrt{R_X^2 + R_Y^2 + R_Z^2} \cdot \sqrt{R'^2_X + R'^2_Y + R'^2_Z}}$$

It follows from Fig. 4 that calculation of the angular velocity is free of those errors which are inevitable in calculating the linear velocity on a high frequency table since its value is independent of the modulus of the radius-vector. In addition, the units in which the angular velocity are expressed (radians/sec) are universal for ballistocardiographs of any system. The high reliability of the data obtained by calculation of the angular velocity is confirmed by the narrower range of its variations for different subjects ($\pm 15$ per cent in relation to the mean value).

VOL. 5 1960

It follows from Table 2 that the angular velocity, as with the linear, varied for different stages of the cardiac contraction. However, the character of these changes differed. Thus, the maximum angular velocity was acquired by the body in the $H_v - I_v - J_v$ intervals, i.e. in the period of action of the reactive forces connected with tension of the ventricles and the start of ejection of blood into the vessels from the heart. The angular velocity differs from the normal in pathological conditions.

TABLE 2. MEAN VELOCITY OF DISPLACEMENT OF HUMAN BODY (LINEAR AND ANGULAR)

| VBCG Intervals | $G_v - H_v$ | $H_v - I_v$ | $I_v - J_v$ | $J_v - K_v$ | $K_v - L_v$ | $L_v - M_v$ | $M_v - N_v$ |
|---|---|---|---|---|---|---|---|
| Duration intervals, sec ($\Delta t$) | 0·05 | 0·06 | 0·09 | 0·11 | 0·08 | 0·06 | 0·08 |
| Change of radius-vector in arbitrary units ($\Delta R$) | 24 | 87 | 260 | 302 | 119 | 36 | 93 |
| Angle ($\varphi$) between radius-vector of main VBCG points in radians | 1·22 | 2·02 | 3·05 | 2·77 | 1·55 | 1·80 | 1·85 |
| Linear velocity in arbitrary units | 480 | 1450 | 2888 | 2827 | 1487 | 600 | 1162 |
| Angular velocity in radians/sec | 24·3 | 33·7 | 33·9 | 25·2 | 19·4 | 30·0 | 23·1 |

In this paper we shall not discuss such physical values as force, energy, etc. since vectorballistocardiography conducted with high frequency tables is ill suited for these purposes. It may be assumed that the use of ultra-low frequency ballistocardiographic tables (see [17, 18]) will be more rewarding for vectorballistocardiographic investigations.

In conclusion, we note that calculation of all the quantitative indices incorporating the values of body displacement must be made from the three-dimensional or, with certain assumptions, from the frontal vectorballistocardiogram. It may be assumed that the discrepancy between the values of the stroke volume computed from the ballistocardiographic formula and the values determined in patients from Fick's principle is partly due to the fact that the displacement was computed only from the spikes of the longitudinal ballistocardiogram.

## SUMMARY

(1) The vectorballistocardiogram constructed from the longitudinal, lateral and dorso-ventral ballistocardiograms is a time curve of the displacement of the human body relative to a nominal "position of rest", i.e. the position at which no forces act on the body.

(2) The vectorballistocardiogram is a complex curve which may reflect a positive (clockwise arrow), negative (counter-clockwise) or mixed direction of movement of the body in space. The greatest longitudinal displacements of the human body occur

during ventricular systole. In pathological conditions the transverse displacements are relatively increased.

(3) The linear velocity of the body displacements computed from the time curve is not an accurate index. The angular velocity of the displacement is a value comparable for different ballistocardiographic systems. This index is free from the defects associated with the mode of recording body displacement.

*Translated by* A. CROZY

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# PHOTOREACTIVATION OF PLANT CELLS*


A. P. DUBROV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow


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SINCE 1949 particularly close attention has been centred on the antagonistic action ultra-violet and visible light in relation to the effects produced by ultra-violet irradiation of living organisms. Much research has been done on this phenomenon which has been termed photoreactivation but most of it has been concerned with bacteriophages, protozoa, bacteria and, to a far less degree, plant specimens [1]. Apparently,

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the complexity of the phenomena of photoreactivation in plants, in which these processes may be accompanied by the usual photosynthetic processes, has led investigators to adopt as a criterion of photoreactivation the appearance of morphological differences in irradiated and reactivated objects (browning of leaves as a result of destruction of chlorophyll, appearance of necroses on inoculation with the tobacco mosaic virus, etc.). In a number of cases this causes serious misgivings since it is not clear whether the photoreactivation occurs in the same cells which have been exposed to the initial ultra-violet irradiation and, consequently, to radiation damage or whether the subsequent illumination by the visible part of the spectrum has contributed to normal growth and development of cells which have been protected by the surface layer of cells.

In view of this, we concentrated on a study of the processes of photoreactivation in the very cells previously irradiated by a broad band of ultra-violet radiation. The main criteria of the state of the cell were the values of the isoelectric points (i.p.) of the cell colloids, the plasmolytic and sorption properties and the motion of the cytoplasm determined from the movement of the spherosomes.

VOL
5
196

## MATERIAL AND METHODS

A description of the technique has been published by us previously [2]. We shall only refer to the general conditions of irradiation and reactivation. The investigations were made on the epidermal cells of the inner side of the scale of the onion. *Allium cepa* (variety "Gribov Annual"). The control and experimental material in all the investigations were sections taken from the central zone (8 mm) of the third scale, counting from the outside. The source of ultra-violet radiation was a SVD-120 A lamp, the intensity of radiation was determined by a UF-2 ultra-violet dosimeter [3], irradiation with ultra-violet light being carried out for 5 to 180 sec. For reactivation we used the radiation of 30 W "white light" fluorescent lamps and also PRK-2 and SVD-120 A lamps with various light filters (KS 13, OS 11, ZhS 16, ZhS 12, ZhS 4) of 5 mm thickness. The flux of radiation for the sources of reactivation was determined by the pyrometer of Ianishevskii and was 0·008 W/cm$^2$.

## EXPERIMENTAL SECTION

### *A. The i.p. of the cells*

As has been shown, ultra-violet radiation results in change in the electrical charge of the cell colloids, namely, in a shift in the pH of the i.p. to the acid side [2]. In this work change in the i.p. of the cell colloids was investigated with ultra-violet radiation followed by reactivation, immediately after irradiation. The results of the experiments to determine the i.p. of the cytoplasm and the nucleus with dyes (methylene blue and acid fuchsin) for a number of different doses are given in Table 1 and represented graphically in Fig. 1. In all cases the observations were made 24 hr after irradiation on material kept in uniform conditions (in the dark at +20°).

As Table 1 shows, recovery of the normal electrical properties of the protoplasmatic colloids was not complete in contrast to that for the cell nucleoproteins. A possible

TABLE 1

| Without irradiation | | Ultra-violet irradiation* | | | |
|---|---|---|---|---|---|
| Controls | | Darkness | | Reactivation | |
| Cytoplasm | Nucleus | Cytoplasm | Nucleus | Cytoplasm | Nucleus |
| A† 4·1–3·9 | 3·5–3·4 | 3·3–3·5 | 2·9–3·1 | 3·7–3·9 | 2·8–3·2 |
| B 4·0 | 3·45 | 3·40 | 3·00 | 3·80 | 3·40 |

* Irradiation dose $8 \times 10^6$ erg/cm$^2$; time of reactivation 15 min.
† (A) limits of change in isoelectric zone. (B) mean value.

explanation is that the nucleus is the site of the initial photo restorative reactions, confirmed by the investigations of the role of the nucleus and cytoplasm during restorative reactions [4]. Reference must also be made to the fact that with use of strong

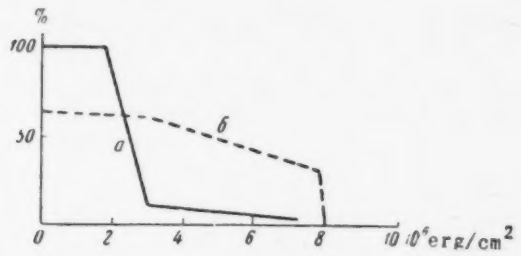


FIG. 1. Change in pH of isoelectric point in irradiated and reactivated cells. (a) irradiated; (a) reactivated cells.

radiation doses ($\approx 10^7$ erg/cm$^2$) the reparative processes in the irradiated cells did not occur (see course of curve (b) in Fig. 1).

*B. Plasmolytic properties of cells*

The plasmolytic test is one of the most important methods of studying both the protoplasmatic boundary layers and the bulk of the cytoplasm on subjecting the cell to various physical factors. It also permits very precise determination of changes the properties of the tonoplast and the plasmolemma in response to various doses of ultra-violet radiation acting on the epidermal cells of *Allium cepa*. As our investigations show, with only low doses of ultra-violet radiation ($0{\cdot}5–1{\cdot}5 \times 10^6$ erg/cm$^2$) differences in the properties of the protoplasts of the irradiated and unirradiated cells were very pronounced. When the irradiated sections were placed in 0·6 M $KNO_3$ solution they were completely plasmolysed within 30–50 sec whereas complete plasmolysis of the unirradiated cells took 5–8 min. In order to define the role of photoreactivation in the resotration of the plasmolytic properties of the cells we followed the course of plasmolysis in the irradiated and reactivated cells on irradiation with various doses of ultra-violet. We investigated the character of the responses 24 hr after irradiation: the control (unirradiated), irradiated and reactivated sections of the

epidermis were placed in a solution of plasmolytic agent (0·6 M $KNO_3$) for 5 min, followed by a count of the plasmolysed cells, the results are shown in Fig. 2. Comparison of curves (a) and (b) shows that the reactivation illumination plays a definite role at doses not lethal for the cell during the 12 hr after irradiation (3·0–4·0$\times$ $10^6$ erg/cm$^2$); at high doses (8·0–9·0$\times 10^6$ erg/cm$^2$) it was ineffective. Apparently,

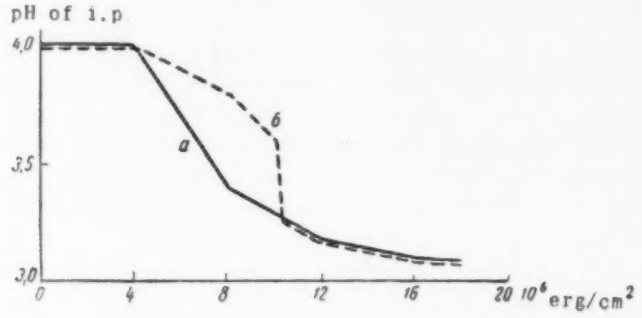


FIG. 2. Course of plasmolysis in irradiated and reactivated cells. (a) irradiated; (b) reactivated cells. Ordinate, percentage of plasmolysed cells.

it may be assumed that with large doses of ultra-violet radiation heavy accumulation of organic peroxides or other toxins occurs, such as to prevent subsequent reactivation illumination from removing their lethal effect [5]. Moreover, the slight degree of recovery from less damaging doses and the absence of effective reactivation for large doses of ultra-violet radiation must be attributed to the low general level of the metabolic reactions in the epidermal cells.

*C. Sorption properties of the cells*

To study the changes in the sorption properties of the cells on ultra-violet irradiation and photoreactivation we made use of the phenomenon, known in fluorescence microscopy as the so-called concentration effect, arising from the fact that with change in the concentration of the fluorochrome the colour of the fluorescence in the cell changes [6]. We would recall that in normal (unirradiated) cells of the inner epidermis of the scale of the onion, *Allium cepa*, placed in buffered solutions of the fluorochrome acridine orange (pH 7·0–7·3) there is heavy accumulation of the dye in the vacuoles so that they show up bright red on inspection in the fluorescent microscope. But as was shown by us earlier, ultra-violet irradiation causes a considerable change in the sorption properties of cells, in particular, in the ability of the plasma to allow penetration and fixing of the fluorochrome [2]. The result is that the uptake of acridine orange by the vacuoles of the irradiated cells is disturbed. We used the accumulation of acridine orange in the vacuoles to indicate the processes of damage and reactivation. On the basis of the concentration effect of acridine orange we investigated the spectrum of action for photoreactivation and the effect of temperature during reactivation illumination (Fig. 3).

*1. The spectrum of action for photoreactivation.* The problem of the spectrum of action for photoreactivation has still not been fully investigated; for bacteriophage it is 315–480 mμ; *E. coli* 375 mμ; sea urchin eggs 300–500 mμ. The photoreactivation of cells irradiated with ultra-violet ($6 \times 10^6$ erg/cm$^2$) was carried out by us by illumination through various glass filters (80×80 mm, 5 mm thick): KS 13, transmitting rays with a wavelength $\lambda > 6200$ Å, OS11 — $\lambda > 5800$ Å, ZhS 16 — $\lambda > 5200$ Å, ZhS 12 $\lambda > 4200$ Å and ZhS 4 $\lambda > 3800$ Å. Besides these filters the SZS 18 and SS4 filters were occasionally used and as Fig. 4 shows, the filters made it possible to gain a much fuller idea of the limits of the spectrum of action. The results given by the various filters, in the study of the spectrum of action of photoreactivation, are shown in Table 2, in which the criterion of reactivation is the above-described ability of the cells to take up acridine orange in the vacuoles. The results make quite clear the effect of rays shorter than 420 mμ, however, in relation to our specimen, it is hard to postulate any specific system as acceptor of the frequency range isolated (we would note in passing that the absorption maximum of the porphyrins is at wavelength 436 mμ).

VOL. 5 1960

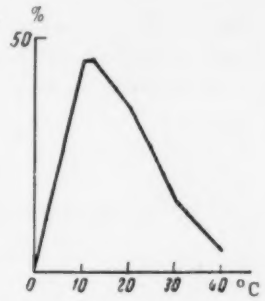


FIG. 3. Effect of temperature on reactivation of uptake of acridine orange by cell vacuoles; ordinate, percentage of controls, abscissa, temperature.

*2. Effect of temperature.* To study the temperature effect on photoreactivation, tubes containing the preparations of the irradiated epidermis were placed during illumination with reactivation light in Koch dishes containing water maintained at a suitable temperature. At the end of reactivation (10–15 min) the preparations were transferred to tubes with water at normal room temperature (+18°).

The data on the photoreactivation at 0° were obtained by us by irradiation and reactivation of sections kept throughout on the surface of a piece of melting ice; later these sections were also transferred to the dark where they were kept on a water surface in tubes at room temperature. As can be seen from Fig. 3 indicating the effect of temperature on the reactivation of the sorption process, the maximum effect (highest percentage of cells with normal uptake of acridine orange) occurred between +10 and +12°. It should be noted that the general course of the curve contrasts with the results obtained for the effect of temperature on photoreactivation of haricot leaves [1]. In the experiments of these workers the total percentage of necrotic foci appreciably

TABLE 2

| Series No. | Percentage of cells with normal fluorochrome uptake | | | | | | |
|---|---|---|---|---|---|---|---|
| | Before irradiation | 24 hr after irradiation | | | | | |
| | Controls | Darkness | Photoreactivation | | | | |
| | | | KS13 | OS11 | ZhS16 | ZhS12 | ZhS4 |
| 1 | 100 | 2 | 1 | 4 | 3 | 15 | 40 |
| 2 | 100 | 6 | 3 | 1 | 2 | 20 | 35 |
| 3 | 100 | 4 | 2 | 7 | 1 | 25 | 55 |
| Average | 100 | 4 | 2 | 4 | 2 | 20 | 43·3 |

fell with rise in temperature (80 per cent at 10°, 48 per cent at 30°). Here, perhaps a role is played by the specific effect of temperature on reactivation of the various processes.

*D. Movement of protoplasm*

The effect of photoreactivation on the translocation of spherosomes in the cells was tested in two varieties of onion "Gribov Annual" and "Danilov". In both cases as was to be expected, the movement was the most sensitive sign of the vital activity of the cell [7, 8]. In the cells of the upper epidermis of the onion scale we could clearly distinguish both the sliding and circulation type of motion of the protoplasm, such

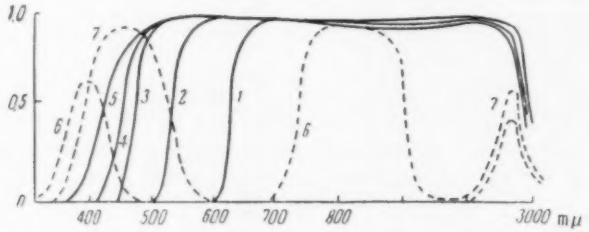


FIG. 4. Spectral curves of transmission of glass filters (thickness 5 mm). (1) KS13; (2) OS11; (3) ZhS16; (4) ZhS12; (5) ZhS4; (6) SS4; (7) SZS18.

observations being best made in a dark field [7]. For greater statistical reliability of our findings, the calculations of the change in velocity of the protoplasm given here were made on large layers of protoplasm and not on individual plasmatic rods as the movement of the spherosomes in the rods often does not reflect the general character of the motion. The velocity of motion of microsomes in normal unirradiated cells at $\pm 20°$ is quite high $7{\cdot}19 \pm 0{\cdot}05$ μ/sec. On irradiation with low doses of ultra-violet ($0{\cdot}5 \times 10^6$ erg/cm$^2$) reduction in the speed of the circulation motion was observed whereas the sliding movement remained unchanged. A further increase in the dose led to progressive retardation of movement. Thus, a dose of $1{\cdot}5 \times 10^6$ erg/cm$^2$ led to arrest of movement after 24 hr and a higher dose ($2{\cdot}0$–$2{\cdot}5 \times 10^6$ erg/cm$^2$) prevented movement immediately after irradiation. Further increase in the doses of ultra-violet

radiation resulted in marked changes also in the sliding movement; 1 hr after irradiation with doses of the order of $3{\cdot}5\times10^6$ erg/cm$^2$ the paths travelled by the spherosomes were shortened with increase in the intensity of Brownian motion, which could be particularly well observed in the corners of the cells where by this time a slight contraction of the vacuoles had occurred because of swelling of the protoplasm. The reactivation of the cells (filter ZhS4) caused considerable changes in the character of cytoplasmatic movement. Whereas in the irradiated cells the velocity of motion of the spherosomes at doses of $1{\cdot}5\times10^6$ erg/cm$^2$ fell to $4{\cdot}83\pm0{\cdot}08$ $\mu$/sec in reactivated cells it persisted at the level characteristic of normal cells (6·91 $\mu$/sec). In contrast to the irradiated cells, the reactivated cells maintained the motion of the spherosomes at $6{\cdot}5\pm0{\cdot}16$ $\mu$/sec even for a dose of $2{\cdot}0\times10^6$ erg/cm$^2$, a dose which would usually lead to immediate arrest of motion. However, with a further increase in the dose of ultra-violet radiation, reactivation of motion was not observed.

VOL. 5 1960

## DISCUSSION

In the copious literature devoted to photoreactivation there are references to the most diverse relations in different specimens both with respect to the general level of reactivation and its effect on the particular properties of the cells. Photoreactivation may be completely absent (see Hill and Rossi [9], who did not obtain reactivation in relation to survival of T1 phages irradiated in the dried form, and a number of other papers) or the restorative processes may occur only in relation to certain properties and not affect others (see [10]). In this reference the efficacy of photoreactiva-

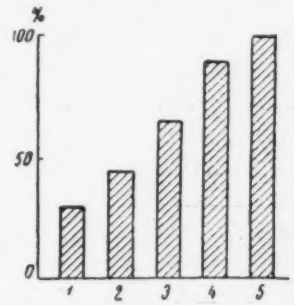


FIG. 5. Schematic representation of restoration for different cell characteristics. (1) plasmolysis; (2) uptake of fluorochrome by vacuoles; (3) i.p. of cytoplasm; (4) cytoplasmatic movement; (5) i.p. of nucleus. Ordinate, degree of recovery as percentage of controls.

tion in nullifying the retarding effect of ultra-violet on division of the eggs of *Arbacia* (but not on the processes of cytolysis, activation of eggs, etc) is demonstrated. Comparison of the results of our experiments suggests a similar conclusion as illustrated by the diagram in Fig. 5 which shows the degree of recovery of the various properties of the cells investigated as a percentage of the responses and properties of normal unirradiated cells. It is accordingly of interest to compare the results of reactivation in respect of the electric charge and the properties of the boundary layers of the pro-

toplasm (Fig. 1 and 2). The most striking point revealed by this comparison is the much higher sensitivity of the plasmolemma to the damage, being present at doses much less ($1{\cdot}5\times10^6$ erg/cm$^2$) than the doses producing just as marked change in the i.p. of the cell ($8{\cdot}0\times10^6$ erg/cm$^2$). This was also confirmed in experiments on the up take by the cells of acridine orange; at low doses of radiation ($0{\cdot}5$–$1{\cdot}5\times10^6$ erg/cm$^2$) which causes no injury during several days, normal accumulation of the cations of the fluorochrome occurred and only placing these cells in plasmolytic agent (0·6 M $KNO_3$ solution) enabled us to note a change in the properties of the boundary layers of the protoplasm. The above noted rapid onset of plasmolysis in the irradiated cells (see section "*B*") can be considered, on the one hand, as the result of the reduced viscosity of the protoplast and, on the other, to be confirmation of increasing water permeability of the irradiated cells [11, 12]. A further interesting fact is that recovery of the electrical properties of the nucleoproteins and movement of the cytoplasm occurred much more fully and thoroughly than did recovery of the other properties and processes. However, account should be taken of the large difference in the value of the threshold doses ($2{\cdot}0\times10^6$ erg/cm$^2$ and $8{\cdot}0\times10^6$ erg/cm$^2$) and a very interesting aspect is the high degree of recovery of motion whereas this property was the most sensitive to ultra-violet radiation. If we consider the data on the effect of temperature during reactivation we may conclude that rise in temperature does not increase the relative level of reactivation in the test object although in the view of some investigators high temperature coefficients should be expected for the processes of reactivation [13]. In our experiments the best result was given by temperatures from +10 to +12°. Apparently, increase in the reactivated material of the number of cells with normal uptake of acridine orange after photoreactivation at +10 to +12° is evidence not of the processes of reactivation proper but of considerable retardation of the processes of damage due to ultra-violet radiation, and, in particular, formation of a smaller number of free radicals and protein break-down products. It is also obvious that the incomplete restoration of the i.p. of the cytoplasm is due to the fact that the i.p. is dependent not only on the presence of free ribonucleic acid but on the composition of the proteins and (a point we should particularly like to stress) the structural state of the protein complex [14]. Evidence of this is possibly provided by the profound changes in the cytoplasm confirmed by work in which escape of Ca and formation of oxalates on irradiation of cells with ultra-violet rays has been noted [15] and formation of holes and breaks in the cytoplasm [11], etc. Attention must again be drawn to the rôle of total metabolism in the cell which may significantly determine the general course and the final results of reactivation; this apparently must be reckoned with on examining the photoreactivation phenomena in cells of the epidermis of the onion scale — an organ in the resting state with inhibited metabolic processes.

## SUMMARY

(1) Study of the photoreactivation of cells of the epidermis of the scale of the onion *Allium cepa* irradiated by a broad band spectrum ultra-violet shows the presence of a variable degree of recovery of the cell properties and processes.

(2) The fullest recovery was noted for the isoelectric point of the nucleus and the motion of the protoplasm for different threshold doses of radiation ($8{\cdot}0\times10^6$ erg/cm$^2$ and $2{\cdot}0\times10^6$ erg/cm$^2$ respectively).

(3) In the epidermal cells irradiated with a dose above the threshold photoreactivation was not observed.

(4) The spectrum of action of photoreactivation is in the region of wavelength 380–420 mμ.

The author wishes to express his deep appreciation to the Scientific Director, Candidate of Biological Sciences, S. V. Tageyeva and Professor M. N. Meisel' for valuable critical remarks and detailed analysis of the manuscript in preparation for the press.

*Translated by* A. Crozy

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# ULTRA-VIOLET FLUORESCENCE OF CERTAIN ANIMAL ORGANS AND ITS CHANGE ON IRRADIATION*

Sh. D. Khan-Magometova, A. V. Gutkina, M. N. Meisel', L. S. Agroskin and N. V. Korolev

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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Recently, a number of papers have appeared on inherent fluorescence in the ultra-violet region of the spectrum detected in certain organic compounds of biological importance and also in animal tissues and plant organisms. Ultra-violet fluorescence

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has been demonstrated in proteins, some amino-acids, [1–5], vitamins and other substances [6].

Brumberg, Meisel', Barskii and Bukhman [7] have studied the ultra-violet fluorescence of a number of animal tissues by means of a special method of microscopy.

Our aim was to obtain the spectra of ultra-violet fluorescence (u.v.f.) for certain organs and to find out whether its character changes under the influence of ultra-violet and X-irradiation. In our experimental conditions, we recorded the spectra obtained from considerable areas of the organs without special regard for their complex tissue and cell mosaic. However, in selecting the objects we primarily investigated organs in the structure of which definite tissue components prevail, namely, the lymph nodes, the spleen, liver, bone marrow, oesphagus and heart muscle. Examination was also made of the blood plasma. As experimental animals we chose white rats (males) selected by weight and age (50 normal and 75 receiving whole-body X-irradiation). Irradiation was from a RUM-3 unit, 0·5 mm Cu+ 0·5 mm Al, distance 30 cm, dose rate 95 r/min. The radiation dose was 1000 r. The organs were extracted immediately after decapitation and either thin sections were prepared from them on a freezing microtome or they were made into a homogenous pulp. The blood was centrifuged in the cold with addition of heparin (heparin does not fluoresce in the ultra-violet region). Only samples of plasma were selected which on comparison on a FEK-M photocolorimeter gave quite similar values. The specimens thus prepared (pulp of organs, blood plasma) were placed in a special quartz cuvette, 0·80 mm in height, and thus a constant thickness of the test object was ensured.

Measurement of the fluorescence was made on a photoelectric microspectrofluorometer designed by Agroskin and Korolev. The source of fluorescence excitation was a DRSh-100 mercury-quartz lamp. The emission of the lamp was spectrally divided by means of a mirror monochromator with a diffraction grating. The rays leaving the monochromator were additionally filtered by a Cl+Br gas filter (for work in the region 260–280 mμ).

The fluorescence spectra were analysed by a similar monochromator through the entry slit of which was transmitted an image of the test object magnified ten-fold by means of a quartz fluorite micro-objective. The intensity of the fluorescence was estimated from the signal at the output of the FEU-18 photomultiplier fixed behind the exit slit of the monochromator. The measurements were made with a mirror galvanometer. The findings were treated in accordance with the spectral sensitivity of the microobjective-monochromator-receptor system.

Already, in the first experiments it was found that the short wave ultra-violet irradiation from the source used by us for exciting fluorescence caused appreciable changes in the u.v.f. spectrum of the organs after a few seconds. There was a considerable fall in the intensity of the u.v.f. and a gradual shift of the maximum toward the long wave region of the spectrum. With a decrease in the intensity of the original maximum (true maximum) there was a rise in that of the new maximum displaced towards the long wave region. This effect of "discoloration", depending on intensive photochemical processes, was observed for all the objects investigated, both those

initially X-irradiated and not irradiated and is represented by the dash-dot curve of Fig. 1.

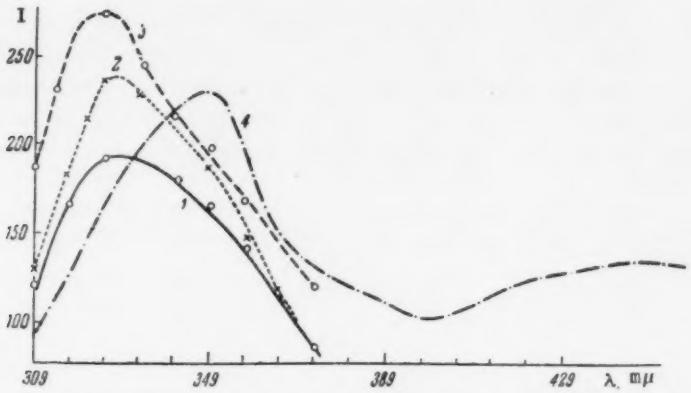


FIG. 1. Fluorescence spectra of blood plasma of control and irradiated animals. (1) controls; (2) 4 hr after irradiation; (3) 24 hr after irradiation; (4) discoloured blood.

It is interesting that the curves characterizing the fall in the intensity of fluorescence were in general identical for all the specimens: the intensity of fluorescence falls by about half in 20 sec (Fig. 2). Therefore, in order to obtain the true u.v.f. spectra for

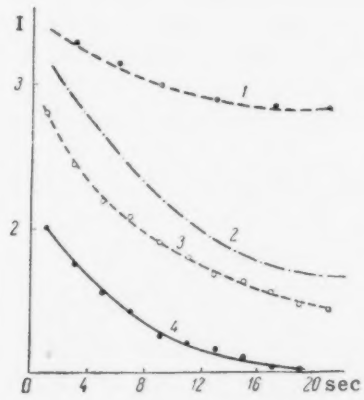


FIG. 2. Reduction of fluorescence maxima under influence of ultra-violet irradiation of tissue. (1) oesophagus; (2) bone marrow; (3) blood, (4) liver.

our specimens we had to fit a special shutter, making it possible to record each point of the spectrum for 0·1 sec and the entire spectrum over 1 sec. During this time no perceptible changes occurred in the spectrum.

The overall u.v.f. of the investigated organs both of normal and X-irradiated animals differed little in their type of spectral curve. The u.v.f. maximum for all the organs

was in the region of 320–330 mμ. The u.v.f. maxima for the aromatic amino-acids are found precisely in this region as are those for some other biologically important substances. It is very likely that the u.v.f. of the various organs is the overall result of combination of the u.v.f. spectra and intensities of the aromatic amino-acids and certain enzymes plus the effect of the presence in larger or smaller amounts of substances quenching fluorescence. It is possible that on investigation at low temperatures a special fluorescence spectrum structure will be revealed, characteristic of a combination of the substances in the given organ. We intend to check this in the future.

TABLE 1. INTENSITY OF ULTRA-VIOLET FLUORESCENCE OF ANIMAL ORGANS FOR CONTROLS AND FOLLOWING X-IRRADIATION (DOSE 1000 R)

| Specimen | Control | 4 hr after irradiation | 24 hr after irradiation |
|---|---|---|---|
| Bone marrow | 1·0 | 1·5 | 1·8 |
| Blood plasma | 1·0 | 1·2 | 1·6 |
| Spleen | 1·0 | 1·2 | 1·5 |
| Lymph nodes | 1·0 | — | 1·2 |
| Liver | 1·0 | 1·0 | 1·05 |

Whereas the type of u.v.f. spectra of the organs of the irradiated animals did not differ from that for the controls, the intensity of this fluorescence for some organs and tissues showed a distinct rise. Our findings are given in the Table (intensity of u.v.f. of normal organs taken as 1). As follows from the Table, the greatest rise in u.v.f. intensity after X-irradiation was observed in the bone marrow and somewhat less in the spleen and lymph nodes, i.e. precisely in the radiosensitive organs.

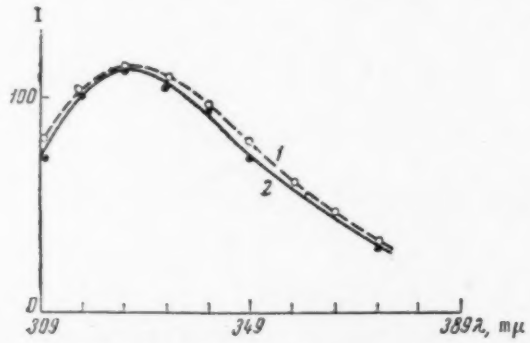


FIG. 3. Fluorescence spectra of liver of control and X-irradiated animals (dose 1000 r). (1) before; (2) after irradiation.

The increase in the intensity of fluorescence depended (within certain limits) on the interval of time elapsing after X-irradiation of the animal. In the liver and other radioresistant organs no changes were detected in the u.v.f. intensity in the irradiated animals (Fig. 3). Of particular interest is the fact that the intensity of the u.v.f. of

the blood plasma regularly rose after X-irradiation of the animals and especially so 24 hr after irradiation. The first changes in intensity of fluorescence were observed only 3–4 hr after irradiation.

It should be mentioned that in all these cases we were unable to discover any significant change in the absorption coefficients, this indicates the higher sensitivity of the fluorescence method of investigation.

In this preliminary communication, it is not possible to analyse the causes and mechanism of the rise in the u.v.f. intensity in radiosensitive organs and tissues of irradiated animals. Possibly, a break occurs in the protein molecule chain with release of cyclical amino-acids and this has the effect of raising the u.v.f. intensity. We cannot exclude the possibility of enhancement of processes associated with migration of energy along the protein molecule. All these problems must be solved by further research.

VOL. 5 1960


## SUMMARY

After whole-body X-irradiation of animals there was a considerable rise in the intensity of ultra-violet fluorescence of radiosensitive organs (bone marrow, spleen, lymph nodes) and blood plasma. The intensity of fluorescence of organs resistance to radiation did not change appreciably.

*Translated by* A. CROZY


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* There is a translation into English of this work.

# TRANSLOCATED EFFECT OF IONIZING RADIATION IN PLANTS*


L. M. KRIUKOVA and A. M. KUZIN

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow


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IN a previous communication [1] we demonstrated that local irradiation of a leaf of 12 to 14-day old plants of *Vicia faba* (10–20,000 r) leads to marked inhibition of

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mitosis both in the stem growing point and in the meristem of the root tips. Inhibition of mitosis from local irradiation of the leaf causes general inhibition of growth and development of the plant. It was assumed that in the irradiated leaf there is formed, or released from a bound state, a substance inhibiting the onset of cell mitosis and that this substance diffuses along the plant, reaches the growing points and thereby reduces the percentage of dividing cells. If this assumption is true then apparently some period of time after irradiation is needed for the formation of the inhibitor in the leaf and its translocation to the growing points. In order to verify this assumption we carried out a series of experiments with removal of the leaf at various intervals of time after irradiation.

The test objects were 12 to 14-day old plants of *Vicia faba*. The plants were placed in a lead chamber and only a single leaf irradiated according to the technique previously described [1].

The irradiation was carried out with X-rays from a RUM-3 unit (without filter, focal distance 21 cm, tube voltage 165 kV, 15 mA), the dose was 10,000 r. The leaf was removed from the plant at various intervals after irradiation — immediately after irradiation and after 4 and 24 hr — or not removed at all. In the control plants (placed in the same lead chamber as the irradiated ones but completely screened) a corresponding leaf was removed after the same intervals.

In all the experiments, 48 hr after the removal of the irradiated leaf a count was made of the number of mitoses in the stem growing points and the tips of the rootlets. The growing points of the plant were fixed with a mixture of ethyl alcohol and acetic acid (3:1). After careful washing the fixed material was stained with aceto-carmine.

In the non-permanent preparations so obtained, the mitotic index was determined. Six sets of experiments were run and in each preparation a cell count was made in 10 fields of view.

The results are given in Table 1.

These findings, in our view, fully confirm the supposition that inhibitory metabolites appear in the irradiated leaf. Removal of the leaf immediately after irradiation completely eliminated the inhibitory effect on mitosis in the growing points. If the leaf was removed 4 hr after irradiation a distinct inhibitory effect was observed in the stem growing point, to where, apparently, the metabolites of the leaf tend to travel. During this short interval only a small portion of the inhibitor reached as far as the root tips (located much further away from the irradiated leaf) and depression of mitosis though discernible was considerably less.

Keeping the irradiated leaf on the plant for 24 hr led to strong inhibition of mitosis both in the sten growing point and the root tips (63 and 40 per cent respectively).

After thus fully confirming the supposition that inhibitors of cell division are formed or released in the irradiated plant leaf, we attempted to isolate the substances from the leaf tissues.

We have previously reported [2] on the extraction from the irradiated embryos of beans, *Vicia faba*, and roots of rye of substances inhibiting mitosis and the develop-

ment of the plants. The experiments reported in the present paper also indicate the formation of inhibitor in cells of the irradiated plant leaf.

TABLE 1. EFFECT OF IRRADIATION OF *Vicia faba* LEAF (1000 R) ON NUMBER OF DIVIDING CELLS IN THE GROWING POINTS

| Conditions | Time of removal of leaf after irradiation | At growing point | | | | | At root tip | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. of cells counted | No. of mitoses | % mitoses | R | % of control | No. of cells counted | No. of mitoses | % mitoses | R | % of control |
| Controls | Immediately | 2659 | 151 | 5·7±0·4 | — | 103·5 | 1353 | 108 | 8·0±0·7 | — | 110·0 |
| Irradiated | after irradiation | 3073 | 181 | 5·9±0·4 | | | 1493 | 133 | 8·9±0·7 | | |
| Controls | 4 hr after | 3465 | 190 | 5·5±0·4 | 4·0 | 62·8 | 1519 | 110 | 7·3±0·6 | 1·2 | 86·3 |
| Irradiated | irradiation | 3598 | 122 | 3·4±0·3 | | | 1325 | 84 | 6·3±0·6 | | |
| Controls | 24 hr after | 3396 | 183 | 5·4±0·4 | 7·5 | 37·0 | 1896 | 149 | 7·8±0·5 | 4·4 | 60·2 |
| Irradiated | irradiation | 3066 | 62 | 2·0±0·2 | | | 1695 | 79 | 4·7±0·5 | | |
| Controls | Leaf not | 2965 | 177 | 6·0±0·4 | 4·0 | 57·0 | 1676 | 150 | 8·9±0·6 | 4·6 | 51·9 |
| Irradiated | removed | 2885 | 98 | 3·4±0·4 | | | 1631 | 75 | 4·6±0·5 | | |

For a more detailed investigation of this problem we irradiated leaves of 12 to 14-day old *Vicia faba* plants with 25,000 r of X-rays. An aqueous extract was obtained from the leaf homogenate 24 hr after irradiation. The proteins were removed from a part of this extract. The non-proteinous, aqueous extract after removal of the reagents was investigated for inhibitor content.

The unirradiated controls were treated in precisely the same way. The extracts obtained — both experimental and controls — had a pH of 5·5, the content of dried substances in the original extract was 600 mg.

In order to detect the inhibitor of cell division, rootlets of *Vicia faba* seedlings were immersed in the solutions obtained (length of rootlets 1·5–2 cm). After immersion in the solution for 24 hr, the plants were washed, specimens prepared from the root tips by the method described above and a mitosis count made. For each preparation 10 fields of view were counted (the data are calculated from the results of counts for ~ 3000 cells). The results are given in Table 2.

One of the many preparations (experimental and control) is shown in Fig. 1.

TABLE 2. PERCENTAGE OF MITOSES IN *Vicia faba* ROOTLET TIPS AFTER 24 HOURS IN TEST SOLUTIONS. (CONTROL PLANTS IN WATER GAVE 5·4–6·0 PERCENTAGE MITOSES)

| Preparation | Solutions from control plants | Solutions from irradiated plants |
|---|---|---|
| Initial extracts (prior to removal of proteins) | 2·8±0·6 | 0·6±0·2 |
| Initial extracts diluted 10 times | 7·2±1·0 | 0·9±0·3 |
| Non-protein extract | 7·9±0·8 | 0 |
| Non-protein extract diluted 10 times | 10·0±1·1 | 0 |

It is clear from the experiments that a weakly inhibitory effect on mitosis was exerted by the aqueous extract from the normal unirradiated leaves. However, this inhibitory effect was completely removed both by dilution of these extracts and by removal of the protein content. In this case we always observed even a certain stimulation of mitosis as compared with plants in nutrient solution or in water (7·9–10 per cent mitoses as against 5·4–6·0 per cent in water).

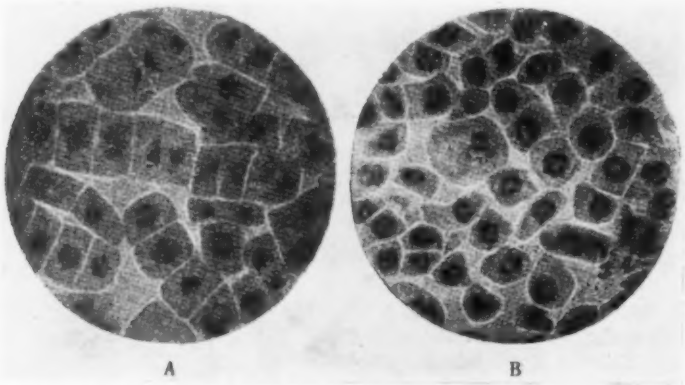


FIG. 1. Tip of *Vicia faba* rootlet kept in initial extract for 24 hr. (A) normal; (B) in solution from irradiated plants.

An altogether different picture was given by the extracts of the irradiated leaves. As Table 2 and Fig. 1 show in this case a considerable suppression of cell division was observed indicating that a substance inhibiting cell division had been extracted from the irradiated leaves.

We are now investigating the chemical nature of this substance.

## SUMMARY

(1) It has been demonstrated that with local irradiation of leaves, inhibitors of cell division are formed or released from a bound state.

(2) The inhibitor is translocated from the irradiated leaves to the growing points and can be detected 4 hr after irradiation. A distinct effect of the inhibitor is manifest after 24 hr.

(3) The inhibitor can be extracted from the irradiated leaves and is of a non-protein nature.

(4) In low concentrations this substance considerably inhibits mitosis in the root tips.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# MECHANISM OF EARLY RADIATION DAMAGE OF BONE MARROW*

S. Ia. Rapoport and S. M. Gasanova

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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The object of the present investigation was to study the rôle of the nervous system in the mechanism of production and development of early radiation injuries, detected by the method of fluorescence microscopy, to the bone marrow.

The work was carried out on male white rats weighing 140–170 g. Irradiation was in the form of X-rays with a dose of 800–1000 r from a RUP-1 unit (180 kV, 10 mA, 0·5 mm Cu+0·75 mm Al, dose rate 40 rev/min).

The method of supravital fluorescence microscopic investigation of the bone marrow devized by Meisel' and Sondak was used and made it possible to observe early changes in the structure of the nucleoproteins [1]. Individual damaged cells and foci of damaged cells — "micronecroses" — appear in the bone marrow in the first few seconds after X-irradiation even with a dose of 100 r [2]. According to these workers the number of micronecroses in the first few hours after irradiation (up to 3–6 hr) gradually rises and then starts to fall off as the damaged cells are resorbed.

In the present work the bone marrow was investigated 2 hr after irradiation, the intensity of damage was expressed by the number of micronecroses per 1 mg of dried bone marrow. The bone marrow of the tibia of the left hind limb was investigated.

## DAMAGE TO THE BONE MARROW ON IRRADIATION OF VARIOUS PARTS OF THE BODY

In studying the role of the nervous system in the mechanism of production of early radiation injury of the bone marrow it was first of all necessary to determine whether this damage on whole-body irradiation is the result of the direct effect of X-rays on the bone marrow or whether it is a secondary phenomenon being the result of irradiation of the whole body. In order to solve this problem damage, to the bone marrow on irradiation of various parts of the body was studied. The results are given in Table 1.

Analysis of the findings in Table 1 shows that a prerequisite for the production of micronecroses in the bone marrow is the direct action of X-rays on the bone marrow. In the bone marrow of the screened limb of the irradiated animal micronecroses were never detected. In this connexion the results of our investigation are in complete agreement with those reported in the literature [2, 3].

* Biofizika **5**: No. 4, 454–460, 1960.

The secondary morphological changes of the bone marrow observed by certain workers [2, 4] in the screened limb were demonstrated at later stages after irradiation and were due, in the view of these workers, to the products of cell disintegration entering the blood.

Table 1

| Conditions | Number of animals | Number of micronecroses per mg bone marrow |
|---|---|---|
| Controls (without irradiation) | 8 | 0* |
| Whole-body irradiation | 68 | 1466±79 |
| Irradiation of one limb | 51 | 777±71 |
| Irradiation with screening of one limb (in the screened limb) | 5 | 0 |
| Irradiation with screening of abdominal region | 8 | 600±126 |
| Irradiation of abdomen | 5 | 0 |
| Irradiation of head | 5 | 0 |

* In the bone marrow of the unirradiated animals no micronecroses were detected, but individual scattered cells were encountered luminescing similar to micronecroses.

On the other hand, the results in Table 1 also indicate the importance of whole-body irradiation in determining the response of the bone marrow to irradiation. Thus, the number of micronecroses in the bone marrow was considerably higher (on average by 1·8 times) on whole-body irradiation (1466±79) than on irradiation of one limb (777±71). A similar picture was observed in a study of the contents of ribonucleic and deoxyribonucleic acids in the bone marrow between the 2nd and 14 th day after irradiation [5]. It was established that the fall in the nucleic acid content is greater and persists for a longer time on whole-body irradiation of the animal than on irradiation of one limb.

The same pattern was also discovered in investigations of the ability of the spleen to initiate regeneration of haemopoiesis after irradiation *in vivo* and *in vitro* [6]. It was established that the mouse spleen loses the ability to regenerate blood cells after whole-body irradiation of the animal with a lethal dose of X-rays if it remains in the body of the irradiated animal but completely retains haemopoietic activity on transplantation to an unirradiated animal.

The following fact was also established: the tissue of the spleen when irradiated *in vitro* with doses exceeding the 100% lethal dose, for the animal, is still capable of forming myeloid tissue on transplantation to unirradiated animals.

Thus, the findings in the literature underline the importance of general disturbances in the development of remote lesions in the haemopoietic organs on whole-body irradiation of the animal and our investigations show the importance of this factor in the development of early radiation damage in the bone marrow.

## EFFECT OF PRELIMINARY INJECTION OF PROCAINE, ATROPINE OR MORPHINE ON THE FORMATION OF MICRONECROSES IN THE BONE MARROW

In a previous investigation [7] we showed that administration of procaine, atropine or morphine before irradiation sharply reduced the number of micronecroses in the bone marrow.

In the present work, in order to clarify the question as to whether these substances act at the very moment of irradiation or in the period directly following it, we studied the effect on early radiation injury to the bone marrow of the injection of procaine, atropine or morphine after irradiation.

All the substances investigated were injected intraperitoneally 5 min after irradiation: procaine, a dose of 1·5–2 ml of 1% solution, atropine, a dose of 1 ml of 0·1% solution and morphine, a dose of 1 ml of 1% solution.

Irradiated animals not injected with test substances served as controls. Tests and controls were always run simultaneously. The results are given in Table 2.

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TABLE 2

| Conditions | Number of animals | Number of micronecroses per mg bone marrow |
|---|---|---|
| Irradiation with 800 r (controls) | 57 | 1390±84 |
| Injection of procaine 5 min after irradiation | 9 | 853±199 |
| Injection of atropine 5 min after irradiation | 11 | 688±183 |
| Injection of morphine 5 min after irradiation | 20 | 479±125 |

As the table shows, all the substances injected after irradiation sharply reduced the number of micronecroses in the bone marrow; procaine by 39 per cent, atropine by 51 per cent and morphine by 66 per cent.

In an earlier work [7] it was shown that on injection before irradiation procaine reduces the number of micronecroses in the bone marrow by 53 per cent, atropine by 48·2 per cent and morphine by 93·7 per cent.

The fact that procaine and atropine injected after irradiation reduced the intensity of damage to the bone marrow to almost the same extent as on injection before irradiation suggests that the effect of these substances on the development of radiation injury to the bone marrow comes about principally in the period following irradiation.

## EFFECT OF PRELIMINARY INJECTION OF PROCAINE, ATROPINE OR MORPHINE ON THE RESPONSE OF THE BONE MARROW TO LOCAL IRRADIATION

In order to understand the mechanism of the protective effect of procaine, atropine and morphine in relation to radiation damage in the bone marrow it was necessary to find out whether it is due to the direct effect of these substances on the cellular elements and thereby on the local radiation reaction.

In order to solve this problem the test substances were injected intraperitoneally 15 min before X-irradiation with a dose of 1000 r to the left hind limb. The results are given in Table 3.

Table 3

| Conditions | Number of animals | Number of micronecroses per mg bone marrow |
|---|---|---|
| Irradiation of limb with dose of 1000 r (controls) | 25 | 730±55 |
| Irradiation of limb after injection of procaine | 11 | 850±95 |
| Irradiation of limb after injection of atropine | 10 | 859±108 |
| Irradiation of limb after injection of morphine | 6 | 17±20 |
| Injection of morphine 5 min after irradiation of limb | 10 | 278±75 |

As Table 3 shows, intraperitoneal injection of procaine and atropine before irradiating one limb exerted no effect on the reaction of the bone marrow as distinct from their effects in whole-body irradiation. It may be inferred from these findings that the protective action of procaine and atropine in relation to damage to the bone marrow on whole-body irradiation is due to prevention of a reaction (or reactions) arising only on irradiation of the whole body.

Only morphine injected before or after irradiation had a pronounced effect on the reaction of the bone marrow to local irradiation.

On injection before irradiation morphine reduced the number of micronecroses in the bone marrow in the case of whole-body irradiation by 93·7 per cent, in the case of local irradiation by 97·7 per cent and on injection after irradiation by 66 per cent and 62 per cent respectively.

Thus, unlike procaine and atropine, morphine exerted a uniform protective action on whole-body irradiation and on irradiation of one limb. It may thus be considered that the protective action of morphine was due to its influence on processes occurring in the damaged tissue itself.

## REACTION OF THE BONE MARROW TO WHOLE-BODY-IRRADIATION (1000 R) AFTER SECTIONING THE SCIATIC NERVE AND INJECTION OF ERGOTAMINE

The reduction in the damage to the bone marrow on injection of procaine and atropine after whole-body irradiation and the absence of any such reduction on injection of these substances before irradiation of a single animal limb provides evidence for postulating the participation of neuro-reflex mechanisms in causing the damage to bone marrow on whole-body irradiation. To check this assumption we studied the response of the bone marrow to whole-body irradiation and irradiation of one limb after sectioning the sciatic nerve and simultaneous pharmacological exclusion of

sympathetic innervation by ergotamine. Our experiments were based on the findings of innervation of the bone marrow [8] obtained in an investigation of serial sections of the whole mouse tibia. These showed that on sectioning of the sympathetic nerve in the inguinal region and high sectioning of the sciatic nerve there is complete disappearance of the nerve fibres accompanying the vessels of the bone marrow and those terminating freely among the cells of the bone marrow. According to these workers, on sectioning the femoral nerve in the inguinal region no signs of degeneration of the nerve fibres are observed in the bone marrow.

In our investigations high sectioning of the sciatic nerve of one limb was carried out 20 min before whole-body irradiation of the animal. In the same period the animal received intraperitoneally 1 ml 0·002% solution of ergotamine. The results are given in Table 4.

TABLE 4

| Conditions | Number of animals | Number of micronecroses per mg bone marrow |
|---|---|---|
| Whole-body irradiation with dose of 1000 r (controls) | 14 | 1776±171 |
| Irradiation of one limb with 1000 r (controls) | 25 | 730±55 |
| Whole-body irradiation after sectioning sciatic nerve and injection of ergotamine | 7 | 713±71 |
| Irradiation of one limb after sectioning sciatic nerve and injection of ergotamine | 9 | 568±90 |
| Whole-body irradiation with exclusion of peritoneal cavity receptors | 11 | 775±79 |

As Table 4 shows, the number of micronecroses in the bone marrow on whole-body irradiation of the animal after injection of ergotamine and sectioning of the sciatic nerve corresponded to the number in the bone marrow after local irradiation of one limb.

Thus, intensification of damage to the bone marrow on whole-body irradiation as compared with local occurs only with retention of the nerve link between the bone marrow and the rest of the body.

A slight reduction in the number of micronecroses on irradiation of one limb after sectioning the sciatic nerve and injection of ergotamine (by 23 per cent) is possibly associated with a reduction in these conditions of the level of metabolic processes and with certain shifts in the haemodynamics.

## EFFECT OF INJECTION INTO THE PERITONEAL CAVITY OF AN OILY SOLUTION OF ANESTHETIZING SUBSTANCES ON THE REACTION OF THE BONE MARROW TO WHOLE-BODY IRRADIATION

Irradiation of the animal with screening of the peritoneal region caused the same lesions of the bone marrow as did local irradiation of one limb (see Table 1). It may be assumed from this that the reflex eliciting intensification of damage to the bone

marrow on whole-body irradiation, as compared with local, is conditioned by stimulation of the receptors of the peritoneal cavity.

In order to solve this problem we studied the response of the bone marrow to whole-body irradiation of the animal with exclusion of the receptors of the peritoneal cavity. This was achieved by injecting into the peritoneal cavity 0·5–1·0 ml of an oily solution of anesthetizing substances (composition of solution: ethyl aminobenzoate 1·0, procaine 0·5, peach oil *ad* 100 ml).

According to the results in the literature [9] this solution produces prolonged local anesthesis. A total of 14 experiments was carried out. The animals were irradiated 2–19 hr after intraperitoneal injection of the anesthetizing solution.

In 3 experiments injection of this solution did not change the usual reaction of the bone marrow to whole-body irradiation. In the other 11 experiments, the number of micronecroses on whole body irradiation of the animal corresponded to the number in the locally irradiated limb (see Table 4).

## DISCUSSION OF RESULTS

The experimental findings given above suggest the conclusion that intensification of damage to the bone marrow on whole-body irradiation as compared with local is due to a neuro-reflex mechanism. This supposition follows from a comparison and analysis of the following facts.

(1) The uniform character of the lesions of the bone marrow on local irradiation of the limb and whole-body irradiation of the animal with subsequent injection of procaine or atropine.

(2) Similar effects of whole-body irradiation of the animal with preliminary sectioning of the sciatic nerve and pharmacologically excluded sympathetic innervation and local irradiation.

(3) Absence of the effect of procaine and atropine injected before irradiation on the response of the bone marrow to local irradiation of the limb as against the presence of an effect on whole-body irradiation.

On the other hand, the findings indicating that irradiation of the animal with screening of the peritoneal cavity or exclusion of the receptors of the peritoneal cavity produces the same response in the bone marrow as on local irradiation of one limb suggest that the initial link in the reflex intensifying damage to the bone marrow on whole-body irradiation is stimulation of the receptors of the peritoneal cavity.

From this point of view the mechanism of the protective action of procaine and atropine in relation to development of damage to the bone marrow on whole-body irradiation must be understood as exclusion of definite links of the reflex arc.

A striking aspect is the special mechanism of the protective action of morphine. While procaine and atropine injected before irradiation did not effect the reaction of the bone marrow to local irradiation at all, morphine, to a uniform degree, prevented damage to the bone marrow both on local irradiation (by 97·7 per cent) and on whole-body irradiation (by 93·7 per cent).

These findings indicate that morphine influences processes in the damaged tissue itself.

The protective action of morphine injected before irradiation in relation both to damage to the bone marrow and to the life span and survival rate of irradiated animals has been noted by a number of workers [10–14].

The majority of workers see the mechanism of the protective action of morphine in tissue hypoxia due to inhibition of the respiratory centre. Such a mechanism of protection implies its incorporation into the irradiation process itself, and is confirmed by the prevention of death of the irradiated animals only if morphine is injected before irradiation. However, this mechanism does not exhaust the effects of morphine in irradiated animals. It has been demonstrated that morphine injected after irradiation considerably attenuates the radiation changes on the permeability of the tissue-blood barriers [14] and considerably reduces radiation damage to the bone marrow. It is possible that this reduction in radiation damage is due to a general decline in the level of metabolic processes in conditions of narcotic sleep.

The mechanism of the protective action of morphine injected before or after irradiation cannot be considered as finally resolved.

VOL. 5 1960

## SUMMARY

(1) Early radiation damage to the bone marrow, detected by the method of fluorescence microscopy, develops only with direct irradiation of the bone marrow.

(2) Early damage to the bone marrow on whole-body irradiation of the animal is much more marked than on irradiation of one limb.

(3) Injection of procaine or atropine before or after whole-body irradiation of the animal lessens the damage to the bone marrow and eliminates the difference usually observed on whole-body irradiation and irradiation of one limb. Injection of these substances does not effect the response of the bone marrow on irradiation of one limb.

(4) Disturbance in the nerve links of the bone marrow or exclusion of the receptors of the peritoneal cavity before whole-body irradiation of the animal also removes the difference in damage to the bone marrow on whole-body irradiation and irradiation of one limb.

(5) The findings indicate that early damage to the bone marrow on whole-body irradiation of the animal is due both to the direct effect of X-rays on the bone marrow and to a neuro-reflex reaction, the initial link of which is stimulation of receptors of the peritoneal cavity.

*Translated by* A. CROZY

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VOL
5
196

# DIPLOID RADIATION GYNOGENESIS IN FISH*†

D. D. ROMASHOV, K. A. GOLOVINSKAIA, V. N. BELIAYEVA, E. D. BAKULINA, G. L. POKROVSKAIA and N. B. CHERFAS

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow, Institute of Pond Pisciculture of the R.S.F.S.R., Moscow

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THE essence of gynogenesis in the broad sense of the term is that the nucleus of the sperm penetrating the ovum is for one reason or another on fertilization excluded from development and the maternal nucleus becomes the nucleus of the zygote. In natural gynogenesis, which is extremely rare and known only for a few animals including certain fish, exclusion of the sperm is a normal process not requiring extraneous action on the sperm. Similar exclusion of the sperm may occur in a number of cases of distant hybridization observed chiefly in fish and amphibia.

Radiation gynogenesis comes about on fertilization of the normal ovum by an irradiated sperm when the damage is sufficiently great for the male nucleus to lose

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its genetic functions. This phenomenon was established and described by Oscar Hertwig and his school and is referred to in the literature as the "Hertwig effect". Investigations on radiation and hybrid gynogenesis in amphibia, instituted by Oscar, Gunther and Paula Hertwig, were further extended in the work of Rugh [1, 2], Rostan [3], Tchou-Su [4], etc.

Little work has been done on radiation gynogenesis in fish. The first investigation was made by Opperman [5] as long ago as 1913 on the stream trout, *Salmo trutta* m. fario. In 1954, Rugh worked with the aquarium fish *Fundulus heteroclitus* [6, 7] and in 1956 interesting material on the grounding was published by Neifakh [8]. A short summary of this work is given in the paper by Golovinskaia and Romashov [9].

On fertilization of the ovum by the sperm the hereditary properties of which are deactivated by the effect of radiation, the sperm essentially gives only an impetus to development, bringing into the ovum the centrosome necessary for subsequent divisions. Since in the ovum there is a haploid set of chromosomes, it is usually inferred that the individual developing as a result of gynogenesis will be haploid ("haploid gynogenesis"); such haploid individuals are not very viable, often deformed and die at various (usually early stages) of development. However, in some of the above studies on amphibia, phenomena of "diploid gynogenesis" have been described and we probably also come across such phenomena in individual cases of distant hybridization of fish.

The prospect of obtaining diploid gynogenetic progeny in fish opens up interesting possibilities not only of a theoretical nature but in the practical sense. For example, the problem arises as to the possibility of sex regulation in fish since it is expected that such gynogenetic individuals will be females. In some cases, for example, in the breeding of sturgeon it becomes very important to obtain a high percentage of females in the progeny because of the high value of the caviare they yield. The use of radiation gynogenesis can also be applied in pisciculture in connexion with the so-called "clone selection". In certain conditions it may be expected that the gynogenetic progeny will completely replicate the genotype of their mother. Since the hybrids (interspecies and interracial) in certain families of fish (sturgeon, salmon, carp) are particularly valuable because of the heterosis they display, persistent repetition of their properties in the progeny is desirable.

On the basis of these considerations, we carried out a special study of radiation gynogenesis in the groundling (*Misgurnus fossilis*), sazán, carp (*Cyprinus carpio*) and Prussian carp (*Carassius auratus* gibelio). The present paper outlines the results of work carried out in 1958 in which the normal spawn of these species were fertilized by irradiated (X-ray) sperm and the phenomena of diploid gynogenesis detected.

For the groundling, these experiments with a very wide range of doses (100–1,000,000 r) were carried out by Beliayeva, Bakulina and Pokrovskaia. Their results are given in Fig. 1.

As the main criteria of radiosensitivity we selected the death rate of the embryos, between the time of fertilization and hatching of the spawn, and the percentage of normal, i.e. not misshapen embryos (of the total number hatched). The appearance

of different types of malformation among the embryos is a very characteristic sign of the damaging effect of radiations [9]. The anomalies in such monsters are quite constant: they are represented by differing degrees of underdevelopment of the anterior, head part of the embryo including lesions of the eyes and underdevelopment of the caudal section, malformation in the region of the heart, pericardium and vascular system, deformation of the body, etc. All these defects are readily distinguishable, often even before the embryo has emerged from the membrane of the roe-corn.

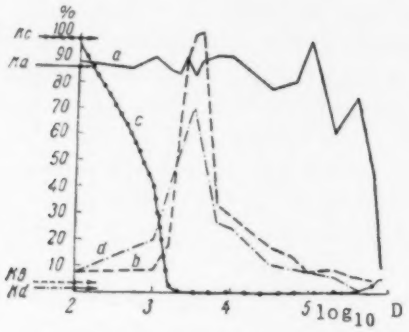


FIG. 1. Radiosensitivity of groundling sperm. Abscissa, dose D, in roentgens, on a log scale; curves: (a) percentage fertilization of roe-corns; (b) percentage mortality of roe-corns from time of fertilization to hatching; (c) percentage of normal embryos among those hatched; (d) percentage of disturbed anaphases and telophases (bridges) at gastrula state. Ordinates, Ka, Kb, Kc and Kd, corresponding values for controls.

Curve (a) (Fig. 1) indicates the percentage fertilization of the roe-corns by the irradiated sperms. Of great interest is the fact that up to a dose of 400,000 r the percentage fertilization changed little, as compared with the controls, for fertilization immediately after irradiation, which is of particular significance for the high doses. For doses longer than 400,000 r, the percentage fertilization fell but even for such a huge dose as one million r there were still individual sperms capable of fertilization.

The effect of irradiation on the embryo mortality (curve (b)) started to make itself felt at doses of 100–500 r when the percentage wastage was about twice as high as in the controls. A very sharp rise in mortality (up to 96 per cent) for the narrow range of doses from 2500 to 6000 r was particularly characteristic. However, a further increase in dose caused a considerable fall in the death rate of the embryos which between 100,000 to 600,000–800,000 r remained at roughly the same low level, only slightly above that for the controls. A similar type of embryo mortality curve was noted by Rugh [1] for the frog *Rana pipiens*, the spawn of which was fertilized by sperm irradiated with 15–50,000 r X-rays.

As for the percentage of normal embryos (curve (c)) it sharply fell from a dose of 100 r upwards and from doses of the order of 2000–3000 r normal embryos completely ceased to appear, this was noted for doses up to 100,000 r. However, on irradiation with very high doses (200,000, 1,000,000 r) there again appeared on hatching quite

normal individuals though in very low numbers. At radiation doses for the sperm of 200,000–400,000 r, they accounted for about 0·5 per cent of the number hatched, or 0·3 per cent of the number of fertilized roe-corns, rising somewhat for the higher doses. We now have a small number of such specimens which are several months old and in which the most careful inspection has failed to reveal the slightest deviation from normal.

In the experiments with carp (Golovinskaia and Cherfas), a limited dose range was tested: 100, 1000, 10,000, 100,000 and 200,000 r. The results were similar to those for the groundling. At a dose of 100 r the harm done to development was comparatively slight and expressed in a minor increase in mortality and a rise in the percentage of deformed embryos. At 1000 r the damage was more clearly marked but not always manifest to the same degree.

VOL. 5 1960

TABLE 1. RESULTS OF EXPERIMENTS ON FERTILIZATION OF NORMAL CARP (SAZÁN) SPAWN BY IRRADIATED SPERM. (RESULTS OF GOLOVINSKAIA and CHERFAS)

| Dose for irradiation of sperm (r) | Total roe-corns (fertilized) | Survival rate of spawn (%) | Percentage of normal embryos among those hatched | Subsequent fate of progeny |
|---|---|---|---|---|
| Controls, 0 | 2356 | 65 | 76 | Normal growth throughout year |
| 10,000 | 1419 | 13 | 0·5 | Died in early larval stages |
| 100,000 | 2602 | 32 | 11 | 17 normal yearlings |
| 200,000 | 431 | 50 | 2 | grown, rest died |

The most damaging dose for the carp was 10,000 r (Table 1 and Fig. 2 (a)). Here, in all the experiments without exception, the mortality of the spawn was extremely high — higher than for any other dose used for the carp. Among the surviving embryos in most cases no normal ones were observed and the deformities were very severe. Individual, apparently normal embryos (on average 0·5 per cent) from the first days of hatching of the spawn showed severe developmental defects and soon died.

At doses of 100,000 and 200,000 r the injurious effect of radiation was lowered: mortality was substantially reduced as compared with a dose of 10,000 r, as shown in Table 1 and Fig. 2 (a). Among the survivors deformed embryos predominated but with less marked deviations from normal. The percentage of normal embryos was, however, comparatively high: for a dose of 100,000 r on average 11 per cent of the number hatched or 3 per cent of the number of fertilized roe-corns. A total of 35 of 126 such "normal" embryos survived to the late larval stages, 17 up to autumn of the same year and 12 at the end of the winter. These fish are completely normal both in outward appearance and behaviour differing in no wise from normal carp of the same age.

According to modern cytogenetic concepts, radiation injury to the organism is caused in the main by disturbances in the chromosome structures. Unfortunately,

we have not yet succeeded in following up the fate of the irradiated sperm during fertilization and the first divisions in segmentation. However, we have at our disposal certain information, as yet preliminary, on the disturbances in the embryonal mitoses in roe-corns fertilized with sperm irradiated with various doses. In the individual roe-corns, at the gastrula stage, a mitosis count was made of disturbed anaphases and telophases (bridges) using the acetocarmine technique of Sverdson. The results for the groundling are given in Fig. 1, curve d. At doses of 100–500 r such disturbances were relatively infrequent, but rose to high values for doses of the order of 2000–5000 r.

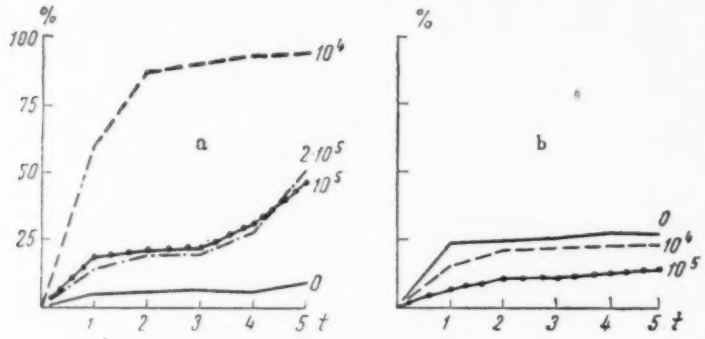


FIG 2. Death during development of carp spawn (a) and Prussian carp spawn for various doses of irradiation of carp (sazán) sperm (b). Abscissa, time (t) in days from moment of fertilization to hatching. Ordinates, percentage mortality of roe-corns from moment of fertilization. Numerals adjacent to curves, doses (r) of radiation for the sperm.

It is obvious that in these cases the largest number of breaks in the sperm chromosomes occurred, leading in the zygote to almost 100 per cent disturbance in embryonal mitoses. With a further rise in dose (of the order of 100,000 r and higher), the percentage of chromosome disturbances fell, gradually approaching that for the controls. Apparently, with such doses, the character of the lesions in the sperm nucleus changes: with increase in dose the breaks in the individual chromosomes become increasingly less significant and their place is taken by severe lesions to the nucleus as a whole (pycnosis, disintegration) and probably this type of damage to the sperm is less destructive to the developing spawn.

A picture similar to that for the groundling was observed by Cherfas for the carp: the number of anaphase and telophase bridges in the embryonal mitoses was about 5 per cent in the controls; on irradiation with a dose of 1000 r it rose to 30–40 per cent and for 10,000 r to 60–70 per cent while at 100,000 r the number of bridges fell sharply almost to the level in the controls (7–12 per cent).

It is clear from the above observation that the more strongly the sperm nucleus is deactivated, the less disruption it causes in the development and the greater the degree of control of development of the maternal undamaged nucleus. It is also evident from our experiments with the groundling and sazán that the appearance at very high

doses of radiation of single individuals normal in morphological structure and viability can be explained only on the assumption that such individuals are diploid organisms ("diploid gynogenesis"). We still do not clearly understand the mechanism whereby doubling of the chromosomes of the ovum comes about in diploid gynogenesis in the groundling and sazán. However, the fact that such diploid individuals are very few in number indicates that this mechanism is insufficiently regulated as suggested by the cytological findings given below.

The next task of our investigations on the radiation gynogenesis in the groundling was to obtain a higher percentage of gynogenetic diploid individuals (Beliayeva). The way to do this, according to extensive research on obtaining triploids in amphibia ([3], [10–17], for example), is to apply shock treatment to the spawn at the moment the nucleus of the roe-corn is passing through the second division of maturation so that by retarding this division the diploid set of chromosomes is retained in the ripe ovum. The shock treatment of the spawn may vary — temperature (hot or cold shocks) or chemical.

VOL. 5 1960

In work with the groundling, experiments have been started with application of cold shocks to the unfertilized or just fertilized spawn (at this time the female nucleus is at the metaphase stage of the second division of maturation). Cooling to a temperature of +1–3° was continued for 3–24 hr. The spawn was fertilized by sperms irradiated with a dose of 200,000 r. The results of individual experiments varied considerably but as a whole the percentage of normal gynogenetic embryos was much higher than in the controls i.e. non-chilled spawn fertilized with sperm receiving the same radiation dose of 200,000 r (Table 2). The best results were given in experiments with cooling of the spawn before fertilization where on average 10 per cent normal individuals were obtained from the number hatched. In individual experiments this percentage reached 13·5 per cent (7–8 per cent of the fertilized roe-corns). These preliminary results can be regarded as quite encouraging.

TABLE 2. RESULTS OF EXPERIMENTS WITH TEMPERATURE SHOCK APPLIED TO NORMAL GROUNDLING SPAWN BEFORE AND AFTER FERTILIZATION WITH IRRADIATED SPERM (RESULTS OF BELIAYEVA)

| Dose of irradiation for sperm (r) | Total roe-corns (fertilized) | Survival rate of spawn (%) | Percentage of normal embryos among those hatched | |
|---|---|---|---|---|
| | | | Mean | In individual experiments |
| | | In normal conditions, from 18 to 20° | | |
| 0 | 3756 | 90 | 89 | 42·0–99·0 |
| 200,000 | 6056 | 80 | 0·3 | 0–0·3 |
| | | With cold shock; from 0 to +2° before fertilization | | |
| 0 | 1973 | 86 | 79·5 | 75·0–86·7 |
| 200,000 | 8764 | 58 | 6·9 | 1·6–13·5 |
| | | With cold shock; from 0 to +2° after fertilization | | |
| 0 | 3199 | 41 | 60·5 | 0–75·7 |
| 200,000 | 10,400 | 37 | 2·9 | 0–16·0 |

Warm shocks were also administered with the just fertilized spawn being exposed to a brief rise in temperature to 33–34°. The percentage of gynogenetic individuals rose slightly and in some experiments reached the figure of 30–36 per cent.

The pattern of behaviour of the female nucleus on fertilization with severely damaged sperm has still to be defined form the paucity of cytogenetic material obtained in the groundling. Inspection of the acetocarmine preparations of the roe-corns fertilized with sperm irradiated with doses of 100,000–200,000 r showed that such roe-corns with respect of their chromosome set may be divided roughly into three groups: (1) mosaic embryos with an inconstant number of chromosomes in the various cells; from less than the haploid to more than the tetraploid; (2) haploid embryos; and (3) embryos with a normal diploid number of chromosomes.

It is interesting to note that at normal temperatures haploid roe-corns are encountered much more often than the diploid together with a large group with an unbalanced number of chromosomes. For cold shocks the picture is different — here diploids appear much more often than haploids but again the largest group is accounted for by embryos with an unbalanced and varying number of chromosomes.

These facts make us adopt as starting point for future work the idea that normal individuals developing from the spawn of the groundling and carp fertilized by heavily irradiated sperm evolve via diploid gynogenesis.

Experiments with *Carassius auratus* gibelio (Golovinskaia and Cherfas) were particularly interesting. As is known this species multiplies by means of natural gynogenesis [13–15]. The Amur line with which we worked is represented solely by females, the spawn of which can be fertilized by the sperm of other species (sazán, carp, goldfish, tench, roach, etc.) yielding unisexual progeny consisting solely of females of *Carassius auratus* gibelio in which no characters of hybridization can be detected. Cytological examination shows that the nucleus of the sperm penetrating the ovum is excluded from development which proceeds solely with the maternal nucleus.

It is natural to assume that on X-irradiation of the sperm of, for example, sazán and fertilization of the spawn of *Carassius auratus* gibelio with such sperm the result would be independent of the dose; in all cases, including the controls, completely normal progeny can be expected. In our experiments the sperm of the carp and sazán were irradiated with two doses: 10,000 and 100,000 r. A quite distinct result was obtained (Table 3 and Fig. 2 (b)); from all the indications (mortality, rate of growth,

TABLE 3. RESULTS OF EXPERIMENTS WITH FERTILIZATION OF NORMAL *Carassius auratus* GIBELIO WITH IRRADIATED CARP (SAZÁN) SPERM (RESULTS BY GOLOVINSKAIA AND CHERFAS)

| Radiation dose for sazán (r) | Total roe-corns (fertilized) | Survival rate of spawn (%) | Percentage of normal embryos of those hatched | Subsequent fate of progeny |
|---|---|---|---|---|
| 0 | 1243 | 68 | 96 | Normal growth throughout year |
| 10,000 | 642 | 76 | 95 | |
| 100,000 | 2112 | 70 | 96 | |

morphological features) the experimental *Carassius auratus* gibelio did not differ from each other. A count of the disturbed mitoses at the gastrula stage in the controls and for the two radiation doses showed that the percentage of chromosome disturbances for these two groups of embryos was identical (4–5 per cent throughout). These results, serving as further striking confirmation of the chromosome theory of hereditry, are self-explanatory.

## SUMMARY

(1) In experiments with groundling and carp, it has been established that X-irradiation of the sperms and fertilization by them of normal spawn gives a degree of damage to the developing embryos (mortality, malformation, disturbances in embryonal mitoses) which increases as the radiation dose rises only within certain limits (for the groundling 2000–6000 r). Beyond this limit the damage is lessened and despite an increase in the dose of radiation a gradual return to normal is observed in terms of mortality and percentage chromosome breaks. Further, although the degree of deformation diminishes, the percentage of deformed embryos remains at a constant extremely high level: single normal individuals make their appearance at very high doses of radiation starting from 100,000 to 200,000 r. The picture of the so-called Hertwing effect is observed in these phenomena.

(2) A maximum degree of damage to the embryos is matched by a maximum number of breaks in the sperm chromosomes. The subsequent mitigation of the damaging effect is associated with destruction of the sperm nucleus as a whole (pycnosis, disintegration) and its genetic deactivation; in such cases control over development is effected solely by the undamaged maternal nucleus (haploid gynogenesis). The appearance of normal, viable carp and groundlings for the strongest radiation doses indicates that in this case there occurs diploid gynogenesis. The low number of such normal individuals shows that the mechanism of diploid restoration is not sufficiently regulated, as is also suggested by the cytological findings.

(3) The application of temperature shocks to the groundling spawn prior to fertilization or just after fertilization with sperm irradiated with 200,000 r raised the percentage of diploid gynogenetic individuals from 0·5 to 7 per cent on average and in individual experiments up to 36 per cent (of the number of embryos hatched). Cytological investigations showed that a high degree of inhomogeneity of chromosome sets is observed in embryos produced by fertilization of normal spawn by sperms irradiated with doses of 100,000–200,000 r: there were isolated individuals with a diploid set of chromosomes, haploids were often encountered, mosaic embryos making up the vast majority. On cold shocks applied to the spawn, diploids were found much more often than haploids, though mosaics still predominated.

(4) Experiments with Prussion carp (*Carassius auratus* gibelio) of a unisexual line showed that the ability to undergo natural gynogenesis enables the spawn of this fish to withstand the destructive action of sperm exposed to ionizing radiations irrespective of the size of the dose employed.

*Translated by* A. CROZY

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VOL
5
196

# PROTECTIVE EFFECT OF HYPOXIA AT VARIOUS RADIATION DOSES*

I. B. BYCHKOVSKAIA and G. K. OCHINSKAIA

Central Research Institute of Medical Radiology, U.S.S.R. Ministry of Health, Leningrad

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No conclusive study has yet been made of the significance of the radiation dose in the protective effect of hypoxia applied at the moment of irradiation. Thus, in an extensive radiobiological literature, there is only fragmentary information on this question from which neither quantitative nor qualitative laws can be established and which greatly handicaps study of the pressing problem of the effect of oxygen lack.

The relation between the protective effect of hypoxia and radiation dose was studied by us with use of a wide range of doses of γ-irradiation, each dose differing from the next by 100 r. In our view, such a choice of doses is particularly advantageous in an attempt to define the quantitative protective effect of hypoxia.

The work was done on male white mice weighing 18–22 g. The animals were irradiated by a GUT-400 telecobalt unit at a dose rate of 13·3 r/min in doses ranging from 900 to 2100 r in steps of 100 r.

Hypoxia was produced by lowering the barometric pressure to 260 mm Hg which corresponds to a 7 per cent content of atmospheric oxygen. According to Dowdy

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and co-workers [1] and our previous findings, such a degree of rarefraction of the air exerts a marked protective effect on mice on irradiation.

In our experiments, the mice were irradiated in a vacuum desiccator of 1·5 l. capacity. The barometric pressure was brought to the required level within 30 min, after which irradiation was carried out. Immediately on termination of irradiation, the pressure was raised over 5–10 min to the original level. The control animals were irradiated in a similar desiccator but with the lid raised for entry of air.

The survival rate of the mice was taken as criterion of the effect of radiation. The mean life time of the dead animals was also taken into account.

Each experiment was repeated not less than 5–7 times and the mean results of all the experimental series are compiled in the table and Fig. 1 which gives the survival rate curves for the mice of the experimental and control groups. Analysis of the results suggests in the first place that for the majority of radiation doses used the survival rate and the mean life time of the mice irradiated with $^{60}Co$ $\gamma$-rays in conditions of hypoxia were higher than in the controls irradiated in normal conditions.

This general conclusion is wholly in accord with the indications in the radiobiological literature [1–7, etc.]. However, a striking conclusion is that the effect of hypoxia for different radiation doses was by no means uniform.

TABLE 1

| Dose groups | Dose (r) | Without hypoxia | | | | With hypoxia | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of animals | No. of animals dead within 30 days | Mean life-span of dead animals (days) | Percentage survival rate at 30 days | No. of animals | No. of animals dead within 30 days | Mean life span of dead animals (days) | Percentage survival rate at 30 days |
| I | 900 | 31 | 14 | 16·0±1·4 | 55 | — | — | | — |
| | 1000 | 40 | 19 | 14·2±1·5 | 52·8 | 48 | 19 | 13·2 ±1·7 | 60·4 |
| II | 1100 | 48 | 43 | 9·5±0·5 | 10·5 | 40 | 18 | 13·1 ±2·9 | 55 |
| | 1200 | 46 | 40 | 8·5±0·6 | 13 | 32 | 20 | 13·14±2·6 | 37·5 |
| | 1300 | 24 | 21 | 8·9±0·7 | 12·5 | 32 | 27 | 8·9 ±1·0 | 15·5 |
| III | 1400 | 36 | 36 | 5·3±0·4 | 0 | 32 | 28 | 10 ±1·2 | 12·5 |
| | 1500 | 42 | 42 | 5·5±0·3 | 0 | 32 | 31 | 7·9 ±1·4 | 3 |
| | 1600 | 36 | 36 | 6·5±0·6 | 0 | 20 | 20 | 6·5 ±0·6 | 0 |
| | 1700 | 28 | 28 | 5·2±0·3 | 0 | 20 | 20 | 6·4 ±0·7 | 0 |
| IV | 1800 | 36 | 36 | 3·9±0·1 | 0 | 27 | 27 | 6·9 ±0·9 | 0 |
| | 1900 | 28 | 28 | 3·8±0·2 | 0 | 18 | 18 | 4·89±1·0 | 0 |
| | 2000 | 28 | 28 | 4·0±0·1 | 0 | 18 | 18 | 4·1 ±0·1 | 0 |
| | 2100 | 28 | 28 | 3·9±0·1 | 0 | 18 | 18 | 3·8 ±0·2 | 0 |

In fact, the effect of hypoxia even varied for doses of $\gamma$-radiation differing from each other merely by 100 r. Thus, while for a dose of 1000 r, the survival rate of the mice irradiated in conditions of hypoxia was not much above that for the controls, the difference in the survival rates for a dose of 1100 r was sharply marked. The effect

of hypoxia for other close radiation doses (1200, 1300, 1400, etc.) also sharply differed.

It was essential to find out the cause of the different degrees of protective effect for the different doses of radiation. In the literature there are references to the fact that the protective effect of hypoxia is most distinctly pronounced for moderate radiation doses, whereas for large and low doses the damage may be so great, or on the contrary, so small, that the protective effect of hypoxia is negligible. However, in our experiments with a large range of gradually ascending doses a new very distinctive relationship between the protective effect of hypoxia and the size of the dose was demonstrated.

In fact, if we follow up how the protective effect of hypoxia varied with increase in the radiation dose (Fig. 1), it will be seen that in the dose range employed there was alternating rise and fall in the effect. Thus, the protective effect of hypoxia, moderately marked for a dose of 1000 r, became very pronounced for doses of 1100 and 1200 r, then virtually disappeared for a dose of 1300 r and again became marked at doses of 1400 and 1500 r. Further increase in the dose again led to a sharp fall in the protective effect of hypoxia up to doses of 1800 and 1900 r when this effect was manifest in a distinct increase in the life time of the experimental mice. Further increase in dose (up to 2000 and 2100 r) again led to disappearance of the protective effect.

Thus, among the gradually ascending doses of $\gamma$-radiation, the following doses could be noted at which a protective effect of hypoxia was distinctly manifest: 1100, 1200, 1400, 1500, 1800 and 1900 r. Minimal protective effect was displayed by doses of 1300, 1600, 1700, 2000 and 2100 r.

In order to analyse this phenomenon, we compared the character of the survival rate curves for the control animals for the doses of $\gamma$-radiation at which in the experimental animals there was a protective effect and for the doses at which it was absent. Figure 2 gives the survival rate curves for mice irradiated with $^{60}Co$ $\gamma$-rays in normal conditions (without hypoxia) in doses rising gradually from 900 to 2100 r. In the figure there are clearly visible four groups, each consisting of curves which virtually merge into each other. The first group of curves reflects the survival rate of the mice irradiated with doses of 900 and 1000 r, the second 1100, 1200 and 1300 r, the third 1400, 1500, 1600 and 1700 r, the fourth 1800, 1900, 2000 and 2100 r. In the first group about 60 per cent of the animals survived, in the second for all doses about 90 per cent of the animals died with a mean life span of 12 days, in the third and fourth groups all the animals died and the mean life span for all doses was for the third group 6 days and for the fourth 4 days. Within each set of curves, a change in dose did not lead to change in survival rate ("saturation" zones).

How do the doses at which the protective effect of hypoxia was marked fit into these groups of doses? From an analysis of the findings in Fig. 2 it follows that these doses occupy a particular position, being the lowest in each group (the first and second). In contrast to this doses of 1300, 1600, 1700, 2000 and 2100 r at which a protective effect was virtually absent occupy the third and fourth places in the groups.

Thus, analysis of the survival curves for the mice provides a simple explanation for the different degree of the protective effect of hypoxia at different radiation doses.

In fact, for doses at the beginning of the group, a reduction of nearly 100 or 200 r sufficed to give the curves a different character and a protective effect thus became distinct. As against this, doses of 1300, 1600 and 2000 r being the third in the group, had to be artificially reduced by not less than 300 r and doses of 1700 and 2100 r by not less than 400 r [*sic*]. It is understandable that for smaller reductions in dose in these cases a protective effect will not be detected, since the curves will remain within the same group.

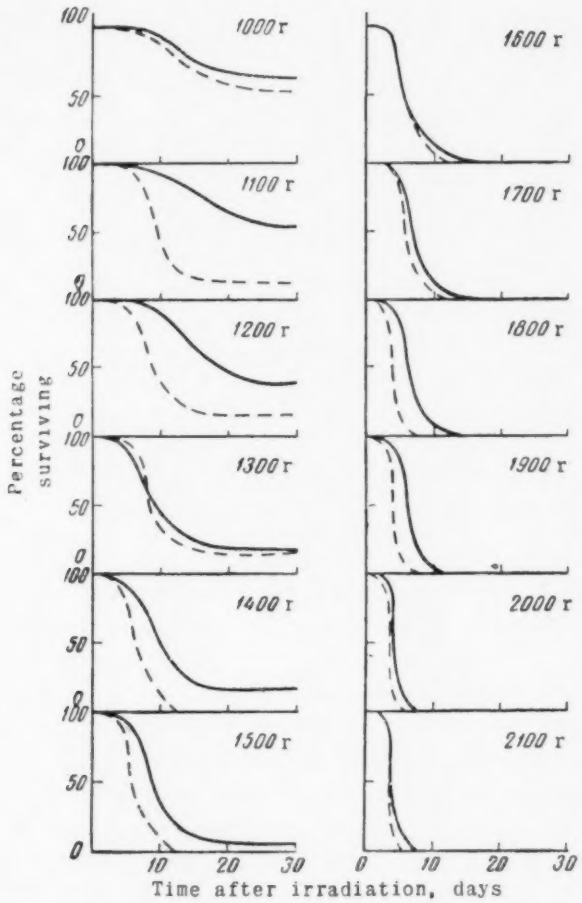


FIG. 1. Effect of hypoxia at moment of irradiation on survival rate of mice. Continuous lines, irradiation in conditions of hypoxia; broken lines, irradiation in normal conditions.

These results hint, in our view, at a new approach to assessing the degree of protection by hypoxia. Firstly, it may be assumed that the protective effect cannot be assessed from a single dose of radiation, as is customary in radiobiological experiments.

In fact, even a sharp increase in the survival rate of the experimental, as compared with the control, animals may result from a fall in dose by only 100 r if the dose was first in its group [*sic*]. At the same time, the magnitude of the divergence of the experimental and control curves (usually taken into account in this work) may depend not on the value of the protective effect but on the zone of "saturation" from which the dose was taken. Figure 2 shows that the distance between the different groups of curves is not uniform. It is thus understandable that the protective effect is most effective for the initial doses of such a "zone of saturation" in which the survival rate curves have the greatest lag behind the preceding group, i.e. in our case for a dose of 1100 r (see Fig. 1).

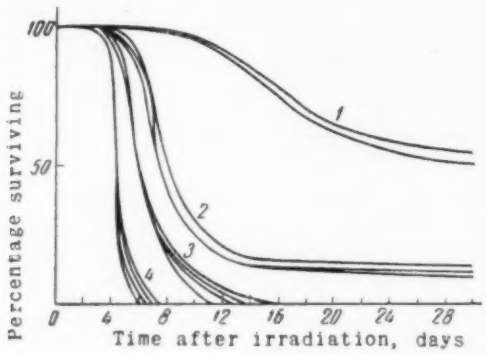


FIG. 2. Survival rate of mice irradiated with $^{60}Co$ $\gamma$-rays in doses of 900–2000 r in steps of 100 r. (1) 900–1000 r; (2) 1100–1200 r; (3) 1400–1700 r; (4) 1800–2100 r.

The question arises as to whether it is possible in our case, in which a large group of gradually ascending doses was used, to make a quantitative analysis of the protective effect of hypoxia.

It was stated above that a protective effect could not be recorded for doses occupying the third and fourth places in a group. Accordingly, it may be asserted that in conditions of hypoxia the size of the "reduction" in the dose for the doses used in our experiments was less than 300 r. In fact, if this value was equal to 300 r, then the survival rate curves for the mice irraduated in conditions of hypoxia with doses of 1300, 1600 and 2000 r (third in the group) would start to approach the survival rate curves for the control animals irradiated with doses 300 r less, i.e. 1000, 1300 and 1700 r and a protective effect would have been visible (Fig. 2). At the same time, in our experiments, the size of the reduction in dose was apparently greater than 100 r for otherwise the survival rate curves for the mice irradiated in conditions of hypoxia with 1200, 1500 and 1900 r (second in group) would have coincided with the survival rate curves for the controls irradiated with doses of 1100, 1400 and 1800 r and no protective effect would have been manifest (Fig. 2). In our experiments a distinct protective effect was noted for doses of 1200, 1500 and 1900 r (Fig. 1).

Hence, it may be assumed that the size in the reduction of dose or doses used in our experiments was less than 300 r and more than 100 r. We shall try to define this value. It is understandable from the preceding remarks that for such a definition it is pointless to study the effect of hypoxia for doses occupying the first and also the third and fourth positions in the group. In fact, it is impossible to find the precise value for the reduction in dose for the first doses in a group since already on reduction of these doses by 100 r the survival curves merge with the set of curves for the preceding zone of saturation consisting of several doses. For the third and fourth doses in the group no protective action is observed at all. We are thus left with the second doses in the groups (1200, 1500 and 1900 r). We shall try to show on which survival rate curves for the control animals are superimposed the survival rate curves for the mice irradiated in conditions of hypoxia with these radiation doses.

Fig. 1 shows that on irradiation of mice in conditions of hypoxia with γ-rays at a dose of 1900 r, the survival rate curve is practically superimposed on the group survival curves for the control animals irradiated with doses of 1700–1400 r, i.e. 200 r and upwards less than the initial dose of 1900 r. The survival rate curves for the control [*sic*] animals irradiated in conditions of hypoxia with doses of 1500 and 1200 r lie somewhat lower than those for the control animals with a dose of 1300 and 1000 r.

Thus, while for a dose of 1900 r, the size of the reduction in dose on irradiation in conditions of hypoxia is of the order of 200 r with possible deviations, for doses of 1500 and 1200 r this value is known to be less than 200 r. It may be assumed that for different initial radiation doses the size of the reduction in dose may vary because of the fact that with irradiation in conditions of hypoxia there is reduced damage not by a constant absolute value but by a definite factor corresponding to a certain hypothetical coefficient of the attenuating effect of hypoxia

$$C = \frac{D_{eff}}{D}$$

where $D$, the initial dose of radiation and $D_{eff}$ the dose reduced under the influence of hypoxia (for our conditions the value of this coefficient according to tentative calculations may be of the order of 0·97–0·98, which corresponds to a decrease in dose of 13–12 per cent). Additional experiments are necessary with use of intermediate doses of radiation (1025, 1950, 1975, etc.) for clarification.

Judging by our results, quantitative changes in the radiation effect do undoubtedly occur on irradiation in conditions of hypoxia. However, as we used only one criterion (survival rate) in our experiments, we cannot deny the possible existence of definite qualitative features in the course of radiation sickness in mice irradiated in conditions of hypoxia with different doses of radiation. This will require further study in experiments with other criteria of the effect of irradiation.

The method of quantitative assessment of the protective effect of hypoxia proposed will be further developed by us. In view of the special morbidity distribution on irradiation (presence of a zone of "saturation") this method, from our point of view, must also be of great interest in investigations of the effect on radiation sickness of various

other factors which mitigate or aggravate the disease. Consideration in radiobiological experiments of the features described seems to us all the more necessary as with use by the experimenter of arbitrary radiation doses, the effect to be expected may not be recorded at all if the doses employed fall within the region of saturation.


## SUMMARY

(1) The doses of $^{60}Co$ $\gamma$-rays used in the work (1000, 1100, 1200, etc. up to 2100 r) in their effect on the survival rate of mice may be broken down into several groups within which change in the size of dose does not lead to change in the survival rate ("zones of saturation").

(2) The protective effect of hypoxia was only noted on irradiation of mice with doses occupying the initial places in the groups (first and second); for the third and fourth doses of the groups the survival rate of the animals irradiated in conditions of hypoxia did not differ from that for the control mice irradiated normally.

(3) The relation between the protective effect of hypoxia and the position of the radiation doses in the group make it posiible to embark on a quantitative assessment of the protective effect.


*Translated by* A. CROZY

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# THRESHOLD REACTIVITY OF VARIOUS REGIONS OF THE HUMAN RETINA TO X-IRRADIATION*


G. K. GURTOVOI and YE. O. BURDIANSKAIA

Institute of Biological Physics, U.S.S.R. Academy of Science, Moscow; Helmholz Institute for Diseases of the Eyes, Moscow


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DOSES of X-rays of the order of a milliroentgen to the human orbital region are sufficient to produce a sensation of light — X-ray phosphene [1, 2]. It has been demonstrated that the primary processes involved here occur in the retina [3]. However,

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it is still not clear in which region of the retina — peripheral rod or central cone — these processes actually take place. Nor was any definite conclusion reached by Lipetz [4] who made a detailed analysis of a large number of papers on X-ray phosphene.

In order to solve this problem, the approach we adopted was to define the threshold radiation doses for X-ray phosphene for persons with various regions of the retina excluded from the process of vision as the result of disease. Experiments on such persons have been described in reference [1], but in this case definite doses were not employed, only two patients were investigated, there was no clear indication of the nature and degree of the lesion in the retina; the authors did not attempt to draw any conclusions from their experiments.

## METHODS

The near-threshold X-ray doses were determined for patients with lesions of the various parts of the retina. The findings for normal conditions published in paper [3] were taken into consideration. Comparison of these doses made it possible to arrive at quantitative conclusions on the threshold reactivity of the various regions and apparatuses of the retina.

The orbital region was irradiated with a beam of X-rays perpendicular to the frontal plane of the face; the beam was of circular cross-section and about 35 mm in diameter. The quality of the radiation used for doses above 10 mr was determined by the voltage on the tube (140–200 kV), for a dose of 2 r no filter was used whereas for doses less than 2 r a 0·5–3·5 mm aluminium filter was introduced. The radiation for doses less than 10 mr was characterized by a tube voltage of 140 kV and a 2·5 mm copper filter.

In order to give a dose of 0·14 sec duration, the special device described in our paper [2] was used. For a dose duration of about 1 sec, the patient had constantly to turn his eyes either towards or away from the beam (by moving the head). The duration of about 1 sec was selected in order to obtain a dose of about 2 r; for the other doses the duration was 0·14 sec. The dose was defined as the product of the intensity of radiation and duration of dose.

A dose range was found in each experiment, i.e. the near-threshold dose at which the subject always perceived light and the near-threshold dose at which he never experienced the sensation. In a number of cases for checking purposes, the X-ray tube was not switched on and the subject had to say whether or not he perceived light.

Each range was determined on the basis of 8–12 near-threshold doses. The dose was regulated by changing the strength of the anode current of the tube. Doses greater than 2 r were not used.

The subjects invited to take part were patients with pigmentosa retinitis with various affections of the field of vision. Eleven eyes of nine subjects were tested. The vision of the subjects is characterized in Table 1. The Figure gives examples of typical fields of vision for three eyes.

## EXPERIMENTAL RESULTS AND DISCUSSION

The results are given in Table 1. They may be divided into four groups: first group — eyes No. 1 and 2 with the central part of the field of vision excluded — central scotoma with a radius of roughly 40′ for which the cone apparatus must be considered as almost completely absent. The dose range here was 2–7 mr, which is very close to the normal (1–2 mr) (see [3]).

TABLE 1. NATURE OF VISION OF SUBJECTS AND THE DOSE RANGE

| Subject | Eye | Eye No. | Nature of field of vision | Figure | Acuity of vision | Dose range, mr | |
|---|---|---|---|---|---|---|---|
| | | | | | | Always sees | Never sees |
| M | Right | 1 | Central scotoma of radius 25–75° | a | 0·01 | 7 | 2 |
| | Left | 2 | Central scotoma of radius 40–50° | — | 0·01 | 7 | 3 |
| N | Left | 3 | Slight reduction in field of vision from periphery, in places by 10° | — | 0·9 | 8 | 2 |
| B | Right | 4 | Vision maintained in central region of retina with radius of about 8° and in band of about 40–60° | b | 0·3 | 8 | 3 |
| B | Left | 5 | About same as for right eye | — | 0·2 | 8 | 3 |
| S | Right | 6 | Vision maintained in central region of retina with radius of about 10–30° | — | 0·03 | 230 | 60 |
| L | Left | 7 | Vision maintained in central region of retina with a radius of about 10–30° | — | | 70 | Not measured |
| N.I. | Right | 8 | Tubular field with radius of about 8° | c | 0·1 (with 5 diopters spectacles—0·4) | 130 | 40 |
| P | Left | 9 | Could not be measured | — | Counts number of fingers at face | Not measured | Not less than 2000 |
| N.I. | Left | 10 | Sees light, but incorrectly identifies its source | — | | Not measured | Not less than 2000 |
| P | Right | 11 | Tubular field with radius about 8° | — | 0·01 | Not measured | 250 |

Second group — eyes No. 3, 4 and 5 with well-preserved rod and cone apparatuses. Dose range was 2–8 mr which coincides with that for the first group.

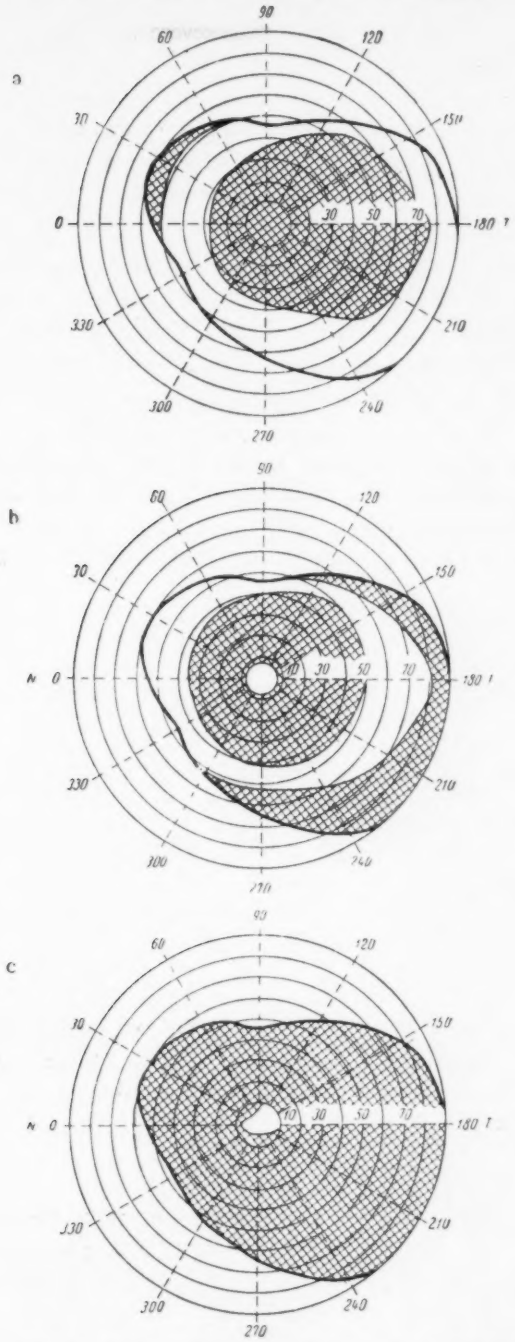


FIG. 1. Field of vision. Normal field of vision enclosed by heavy line. Regions for which patient does not see shaded. (N) nasal side of field; (T) temporal. Numerals show degrees. (a) subject M (eye No. 1 — see Table 1); (b) subject B (eye No. 4 — see Table 1); (c) subject N.I. (eye No. 8 — see Table 1).

Third group — eyes No. 6, 7 and 8 with severe affection of the rod apparatus. Dose range about 40–230 mr.

Fourth group — eyes No. 9, 10 and 11 with very marked affection of rod and cone apparatus. Dose of 250–2000 mr for these eyes was still subthreshold. For the female subject P, doses of more than 250 mr were not delivered so that for the fourth group we must consider a dose of not less than 2000 r as subthreshold.

Table 2 summarizes the results.

It may be concluded from Table 2 that the rod apparatus (and not the cone) is the substrate in which the primary impulse leading to sensation of light under the effect of threshold doses of X-irradiation arises in the normal eye. This follows, firstly, from a comparison of the findings on the dose ranges for the first and second groups. Indeed, the presence or absence of the cone apparatus did not affect the dose range Secondly, this conclusion follows from a comparison of the findings of the second, third and fourth groups since the more marked the involvement of the rod apparatus, the higher the threshold dose. It should be emphasized that the conclusion refers to the comparative reactivity of the rod and cone apparatuses as a whole; it cannot as yet be assumed that the threshold dose for a single rod is less than for a single cone.

VOL 5 196

TABLE 2. GROUPING OF EYES ACCORDING TO DEGREE OF AFFECTION OF ROD AND CONE APPARATUSES OF RETINA AND THE DOSE RANGE

| Group | Eye No. | Degree of affection of retina | Approximate dose range, mr | |
|---|---|---|---|---|
| | | | Always sees | Never sees |
| I | 1 and 2 | Cone apparatus excluded | 7 | 2 |
| II | 3, 4 and 5 | Rod and cone apparatuses well maintained | 8 | 2 |
| III | 6, 7 and 8 | Severe affection of rod and cone apparatuses | 230 | 40 |
| IV | 9, 10 and 11 | Very marked affection of rod and cone apparatuses | Not measured | Not less than 2000 |

The work was done on comparatively restricted material necessitated by the difficulty of obtaining and investigating suitable subjects. More extensive experimental material is necessary for any final conclusions. However, the results obtained suggest the following tentative conclusions.

## CONCLUSIONS

(1) If X-irradiation is delivered to the whole retina of the normal eye then the substrate in which the primary impulse develops (leading to sensation of light with the threshold dose) is the peripheral rod and not the central cone apparatus. In this case the threshold is a dose of about 1 mr.

(2) The region of the retina of radius 10–30° has a higher threshold than the whole normal retina; for this region the threshold dose will be of the order of 150 mr.

(3) For marked degrees of exclusion of both rod and cone apparatuses a dose of 2 r does not produce a sensation of light, i.e. is subthreshold.

(4) The findings may be utilized in clinical investigations of the retina in the case of opaque optical media.

*Translated by* A. CROZY

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# METHODS AND APPARATUS

# PHYSICAL BASES OF RADIOBIOLOGICAL EXPERIMENTS WITH NEUTRON FLUXES *

B. M. ISAYEV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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ONE of the main problems facing modern radiobiology is the investigation of the relative biological effectiveness of various forms of ionizing radiations (r.b.e.) and, in particular, the relative biological effectiveness of neutron fluxes of different energies. It is first necessary in solving this problem, i.e. investigation of the r.b.e., to determine precisely the quantity of energy transferred from the ionizing radiation to the unit mass of object being irradiated, i.e. the absorbed dose.

As is known, the unit of the absorbed dose adopted is the rad, equal to an energy of 100 ergs transferred from the ionizing radiation to one gram of the irradiated substance. Within the scope of the present article we shall not go into the details of the mechanism of the primary process of the biological action and, in particular, we shall not deal with the problem of the mechanism of energy transfer from the ionizing particle to the molecules of the medium.

We shall consider that the absorbed energy can be determined by measuring the amount of ionization produced by the charged particles at a given point in the specimen. We shall assume that the value of the absorbed energy is equal to the number of ion pairs formed in the medium multiplied by the work of formation of one ion pair which is a constant value for a given type of particle and does not depend on the energy of the ionizing particle. Unfortunately, in most radiobiological experiments even with such comparatively "simple" radiations as X- and $\gamma$-rays, calculation of the absorbed dose is neglected and only the "radiation dose" expressed in röentgens determined. However, calculation of the distribution of the absorbed dose over the irradiated object and determination from this of the values of the mean or maximum absorbed dose is the first necessary step in the treatment of a radiobiological experiment. For this reason it is very difficult to compare the findings of different investigators whose experiments have been carried out in different conditions of irradiation.

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In the field of dosimetry of fast neutrons use has been made for quite a long time of the so-called n-unit which is defined as the dose of fast neutrons which will produce in a Bakelite thimble chamber the same ionization as a radiation dose equal to 1 r. Criticism of this unit has been made in a number of papers [1–3]. The use of a Bakelite thimble chamber with a comparatively thick graphite conducting surface and filled with air has led to incorrect definitions of the energy transferred by fast neutrons to biological tissues. The primary effect of this has been oversized values of the r.b.e. in work on the biological effect of fast neutrons.

It should be mentioned that passage from radiation dose to absorbed dose is not always a simple operation. It involves not only determination of the role of scattered radiation, absorbtion of the radiation within the object, etc, (i.e. determination of the "topography" of the dose field in the object) but also a number of mathematical operations.

In the sphere of X- and $\gamma$-rays, measurements on the topography of the dose field are carried out as a rule by means of thimble chamber dosemeters calibrated in roentgens. If the calibration is correctly made, i.e. allowance made for the range of hardness with observance of conditions of electronic equilibrium in the chamber walls, etc then the absorbed dose in the irradiated snbstance is defined by means of the relation [4]

$$A_{\text{sub}} = \psi D, \text{ rad}$$

where $D$ is the radiation dose in roentgens.

The coefficient $\psi$ is equal to the ratio of the absorbed dose in rads to the radiation dose in roentgens measured at the same point.

The value of the coefficient $\psi$ varies for different biological tissues and is dependent on the energy of the photons. As an example in Fig. 1 we give the value of the coefficient $\psi$ for muscle, fat and bone tissue. Along the abscissa we have the energy of the photons in MeV and the ordinates give the values of $\psi$ (rad/r). It is clear from the curves that for the same radiation dose, the absorbed doses may vary a great deal for the different tissues.

When biological specimens are subjected to neutron fluxes, determination of the absorbed dose involves considerably greater difficulties than in X- and $\gamma$-ray dosimetry.

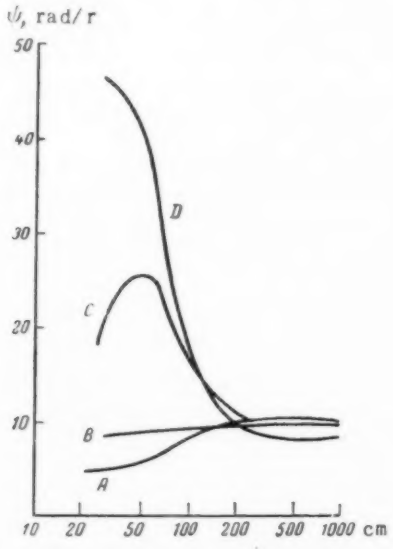


FIG. 1. Relation between radiation dose and absorbed dose. (A) fatty tissue; (B) muscle tissue; (C) bone marrow; (D) bone; Abscissa, quantum energy.

In neutron dosimetry, the problem of determining the tissue dose (by which we mean the absorbed dose in biological tissue or tissue-like substance) of neutrons is complicated by the nature of the interaction of the neutrons and the elements making up of the tissue. On irradiation of any substance with neutrons, the ionizing factor is the secondaries appearing on interaction of the neutrons and the alomic nuclei of the substance. Since biological tissue consists of the light elements (H, C, N, O) it may be considered that neutrons with an energy not exceeding 20 MeV transmit their energy to the nuclei of the atoms via three main processes: (1) elastic scatter, resulting in the appearance of recoil nuclei of hydrogen, carbon, etc; and (2) reactions in the nitrogen nuclei resulting in the emission of a proton with an energy of 0·62 MeV and (3) radiation capture of a neutron by the hydrogen nucleus with formation of the excited nucleus of deuterium. This results in hard γ-radiation with an energy of 2·2 MeV.

The present article will be confined to consideration of neutrons of energy not higher than 20 MeV. Neutrons of energy less than 20 MeV are most often used in radiobiological work and much work has been done on their biological effect.

It is now common to classify neutrons according to their energies, a classification which is somewhat arbitrary and may differ for different workers. This classification was adopted in paper [5]. We have slow neutrons with an energy of less than 1 keV, neutrons of intermediate energies in the 1 to 500 keV range and fast neutrons in the energy range 0·5–20 MeV. Despite the fact that the main interaction between slow neutrons and tissue is elastic scatter, the basic biological action of slow neutrons, including also thermal neutrons with an energy of $\bar{E} = 0{\cdot}025$ eV, is determined by the radiation capture of a neutron by the hydrogen nucleus and by the reaction with the nuclei of nitrogen atoms since the energy transferred on elastic collision is negligible.

TABLE 1. CHEMICAL COMPOSITION OF MUSCLE AND BONE TISSUE AND NUMBER OF ATOMS PER CUBIC CENTIMETRE

| Element | Muscle tissue | | Bone tissue | |
|---|---|---|---|---|
| | Percentage by weight | No. of atoms per g $\times 10^{22}$ | Percentage by weight | No. of atoms per g $\times 10^{22}$ |
| H | 10·2 | 6·15 | 6·4 | 3·85 |
| C | 12·3 | 0·615 | 27·8 | 1·40 |
| N | 3·5 | 0·15 | 2·7 | 0·12 |
| O | 72·9 | 2·74 | 41·0 | 1·54 |
| Ca | 0·2 | — | 14·7 | 0·22 |

As a result of these processes the main ionizing components are the γ-quanta with an energy of 2·2 MeV and protons of energy 0·62 MeV. Since the range of a proton of such energy is $\sim 10^{-3}$ mm, it may be considered that the action of the proton is strictly localized at the site of its origin. We observe a different picture in the case of radiation capture. It is obvious that in a certain unit volume not only may there be absorption of some of the γ-quanta formed in it, but also of those formed at other points of the volume irradiated.

It is also obvious that the number and character of the nuclear reactions is determined by the chemical composition of the biological tissue. The International Commission on Radiological Units has adopted for calculations the chemical composition of the moist tissue shown in Table 1.

The latest and most detailed papers in which tissue doses for neutrons of energy less than 1 MeV are calculated are articles [6] and [7]. Figure 2 gives by way of example the distribution of the components of the tissue dose for thermal neutrons striking perpendicularly the base of a cylinder of 30 cm diameter and 20 cm in height [7]. The ordinates give the values of the tissue dose in rads/neutr/cm$^2$, the abscissa the distance in cm from the base of the cylinder. As these curves show, the value of the "captured" dose is considerably greater than that of the dose produced by the protons via the reaction with nitrogen and is at the surface of the object $5 \times 10^{-10}$ rad/neutr/cm$^2$.

An important contribution to the tissue dose on irradiation of objects with thermal neutrons may be a slight admixture of boron. The capture of a neutron by the boron nucleus leads to splitting of the boron nucleus with the formation of an α-particle and a nucleus of lithium. The reaction occurs in the nuclei of the $^{10}B$ isotope ($^{10}B$ $(n, \alpha)$ $^{7}Li$), the content of which in the natural mixture is about 19 per cent. The total energy of the lithium nucleus and α-particle is equal to 2·5 MeV in 95 per cent of the cases with the α-particle accounting for 1·9 MeV and the lithium nucleus 0·6 MeV. The effective cross-section of the $^{10}B$ $(n, \alpha)$ $^{7}Li$ reaction varies according to the $1/v$ law and $\simeq E^{-1/2}$ ($v$ and E, the velocity and energy of the neutron) and is equal to $\sigma = 114\, E^{-1/2}$ barn if the energy is expressed in electron volts. For the natural mixture of boron, the cross-section of this reaction is about 600 barn and for the pure isotope, $^{10}B$, 4000 barn.

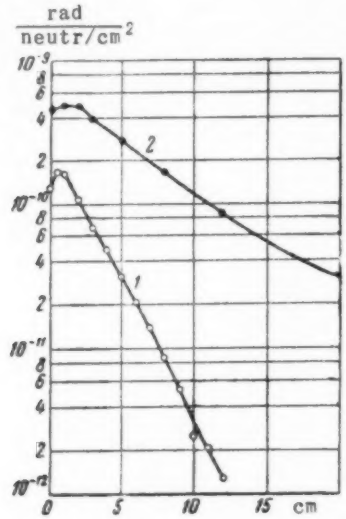


FIG. 2. Distribution of components of tissue dose for thermal neutrons. (1) $^{14}N$ $(n, p)$ $^{14}C$ reaction; (2) $H$ $(n, \gamma)$ D capture.

Calculation shows that in the presence of even low concentrations of boron ($2 \times 10^{-4}$ by weight), the tissue dose from the $^{10}B$ $(n, \alpha)$ $^{7}Li$ reaction is equal to the tissue dose from the protons in the $^{14}N$ $(n, p)$ $^{14}C$ reaction. If we consider that the r.b.e. of the α-particle and lithium nucleus may be higher than for the protons formed in the $^{14}N$ $(n, p)$ $^{14}C$ reaction then the contribution to the biological effect of the $^{10}B$ $(n, \alpha)$ $^{7}Li$ reaction becomes even more substantial.

Artificial introduction of boron, especially of $^{10}B$-enriched boron, may considerably increase the tissue dose from the α-particles and lithium nuclei. These remarks make clear the great importance in work with thermal neutrons of quantitative determination of the boron content of the irradiated specimen bearing in mind that its content may vary within very wide limits. A detailed analysis of this problem is given in paper [8].

Attention must be directed to the fact that the results of the calculations in Fig. 2 refer to a comparatively large mass of irradiated tissue. Obviously, as the mass of the object decreases, the specific contribution of the "captured" dose as compared with the proton dose will diminish. In fact, for very thin specimens we can in general ignore the captured dose since the absorption of the hard γ-radiation in this case will be negligible. This is confirmed in paper [9] where the tissue dose from the protons on irradiation of mice in a reactor was double the tissue "captured" dose, whereas for rats they were equal.

A second but no less important factor is consideration of the albedo, or the coefficient of reflexion of neutrons from the irradiated tissue. The value of the albedo depends on the energy of the neutrons (diminishing with increase in energy) and on the angle of incidence of the neutrons on the surface of the tissue. The value of the albedo, according to paper [10], for thermal neutrons is 0·58 at normal incidence and 0·76 for neutrons incident at an angle of 60°. It should be stated that only in paper [7] were these values of the albedo used, whereas in all earlier calculations and in particular in paper [6] 0·83 was used as the albedo value. The phenomenon of neutron reflexion may considerable alter the size of the tissue dose in relation to the geometry of the experiment. For example, if the object is located in the direct vicinity of the neutron source, for example, the thermal graphite reactor column, the neutron leaving the thermal column may be reflected from the object and again fall into the graphite column from which after undergoing reflexion it enters the object being irradiated, etc. Thus, the conditions of measurement are very important in establishing the relation between tissue dose and neutron flux. Measurements made in the absence of the object are known to give overrated values for the flux.

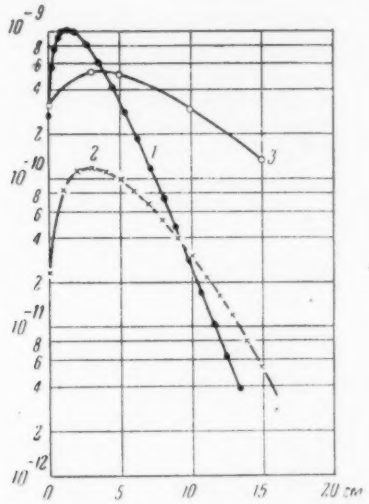


FIG. 3. Distribution of components of tissue dose for intermediate neutrons (neutron energy 240 keV). (1) recoil protons; (2) $^{14}N$ $(n, p)$ $^{14}C$ reaction; (3) H $(n, \gamma)$ D capture.

As the energy of the neutrons increases, an increasing role is played by the recoil nuclei in producing ionization in the tissue. As is known, on elastic collision of a neutron and an hydrogen atom on average half the energy of the neutron is transferred, whereas on collision with carbon, nitrogen and oxygen nuclei the mean energy transferred is several times less. Also noting that per gram of tissue the number of hydrogen atoms is double the number of the heavy nuclei, it may be considered that the chief ionizing effect (over 80 per cent) is accounted for by recoil protons.

The neutrons of intermediate energy (1 keV $<$ E $<$ 500 keV) have been the least studied both in respect of their dosimetric characteristics and their biological effect. At the same time, this region of energies is exceptionally important since intermediate neutrons are usually present in large number in the spectrum of incident neutrons and in turn appear in the irradiated object as a result of the slowing down of fast neutrons. The work done on the dosimetric characteristics of intermediate neutrons [7, 11] shows that from the point of view of biological action they have characteristics intermediate between slow and fast neutrons. Figure 3 shows the distribution of the components of the tissue dose for monochromatic beams of neutrons with an energy of 240 keV, according to paper [7]. For a neutron

energy of 240 keV, ioniziation occurs in the tissue layers close to the surface, in the main through recoil nuclei. The specific contribution of the various components in producing the tissue dose is essentially related to the distance from the source to the irradiated surface. There are no reliable data on heavy recoil nuclei (N, C, O).

The experimental findings on the ionizing power of heavy nuclei with an energy less than 40–50 keV are extremely meagre. In any event it may be assumed that their ionizing power is not great [10] and possibly their basic biological effect is concerned with processes of excitation of the tissue molecules. Since in the region in question, intermediate neutrons, the energy of heavy recoil nuclei on average does not exceed 50 keV (even for a neutron energy of 500 keV), the ionizing effect must be ascribed solely to protons and $\gamma$-radiation.

The main difficulty in the experimental evaluation of tissue doses of intermediate neutrons is that for this region there is no reliable method of detection [11].

A large number of experimental and theoretical studies have been concerned with the dosimetry of fast neutrons [5, 6, 7, 10, 12–20].

It should be pointed out that the theoretical calculation of the tissue doses for various points in the irradiated volume is not a simple task and has only been solved for certain particular cases (semi-infinite medium, filled with tissue-like substance). In Fig. 4 and 5, and in agreement with paper [6], the distribution of the tissue dose for monochromatic fluxes of neutrons with energies of 1 and 5 MeV is shown. The main role in producing the ionizing effect in this energy range is played by recoil protons. With a rise in the energy of the neutrons it is no longer possible to disregard ionization from the heavy recoil nuclei, all the more so since their biological effectiveness may be high. It must again be emphasized that these calculated data can serve only for qualitative assessment of the size of the dose since in actual experiments we have a complex spectrum of neutrons and calculation in this case must be made either for some mean energy or for individual portions of the spectrum. Moreover, in actual conditions in experimental work, as we shall see below, $\gamma$-radiation is present and the dosimetric characteristics of mixed fluxes will differ.

If the mass of the irradiated object is not great (some tens of grams, for example) we can ignore the effects bound up with slowing down of neutrons, considering that the neutron undergoing one collision with any of the atoms passes outside the object. Thus, after the first collision, the neutron "falls out of the game" and no longer takes part in production of the tissue dose. Since we are dealing with fast neutrons, we can disregard the dose from the captured radiation and the proton dose appearing from the $^{14}N\ (n, p)\ ^{14}C$ reaction as the effective cross-section of these reactions for fast neutrons is considerably less than that for elastic scatter. This case is commonly termed the "one-hit dose" or "first-collision dose". The first-collision tissue dose may be readily computed if the neutron spectrum and chemical composition of the object are known.

We shall denote the energy of the incident neutrons by $E_n$ (MeV), the number of hydrogen, nitrogen, oxygen and carbon atoms per gram tissue by $N_H$, $N_N$, $N_O$ and $N_C$ and the fraction of the energy of a neutron transferred (on average) to a hydrogen, nitrogen, oxygen and carbon nuclei by $K_H$, $K_N$, $K_O$ and $K_C$ repectively. It is easy to see that the absorbed dose per incident neutron can be computed by means of the following expression

$$A_{sub} = E_n(K_H \sigma_H N_H + K_N \sigma_N N_N + K_O \sigma_O N_O + K_C \sigma_C N_C) \times 1{\cdot}6 \times 10^{-8} \text{ rad}$$

where $\sigma_N$, $\sigma_H$, $\sigma_O$ and $\sigma_C$ are the effective cross-sections of the elastic scatter for nitrogen, hydrogen, oxygen and carbon. The coefficient $1{\cdot}6 \times 10^{-8}$ converts the transfer of energy expressed in MeV and the energy absorbed expressed in rads. Figure 6 shows the curves relating the absorbed energy of the first collision (in rads) to the neutron energy for muscle and bone tissue. The values of the effective cross-sections are taken from paper [21]. The chemical composition of the muscle and bone tissue adopted for the calculation is presented in Table 1. The other minor constituent elements of the tissue have been disregarded.

The curve in Fig. 6 relating to muscle tissue coincides with that in paper [15]. The values of the tissue dose in bone tissue differ appreciably from that for muscle tissue. The values of the tissue doses

for fatty tissue will be somewhat higher than those for muscle because of its higher hydrogen content (11 per cent). Such a difference in the value of the tissue doses for different tissues must unquestionably be taken into consideration in radiobiological experiments and also in experiments with X- or γ-rays.

As we have stated repeatedly above, in actual radiobiological experimental conditions the use of complex neutron spectra is involved. Besides the fluxes of fast neutrons there are usually present intermediate and slow neutrons and also sometimes a considerable background of γ-radiation. Therefore, it is important to make an analysis of the existing sources of neutrons usually employed in radiobiological experiments. It is also important to do this from the point of view of evaluating the dose rates which can be obtained with different sources.

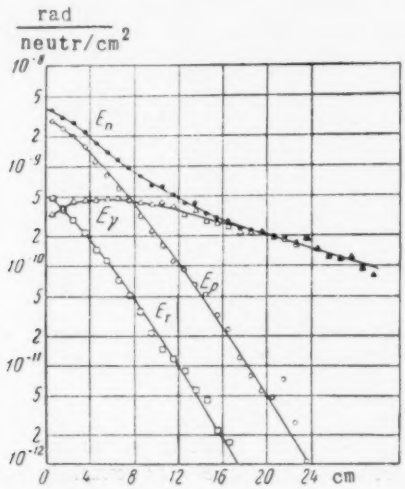


FIG. 4. Distribution of components of tissue dose for neutrons with energy 1 MeV. ($E_p$) recoil protons; ($E_\tau$) heavy recoil nuclei; ($E_\gamma$) H ($n$, γ) D capture; ($E_n$) total dose.

Most research into the biological effect has been with reactors and cyclotrons. However, it must be affirmed that some investigations into the effect of low doses can be successfully made with polonium-beryllium sources. The neutrons in the polanium-beryllium source are formed on bombarding the beryllium with α-particles of polonium ($^9$Be (α, $n$) $^{12}$C). The advantage of the Po-Be source is the very small γ-background since decay of polonium is accompanied by very weak γ-radiation ($10^{-5}$ quanta per decay). The neutron spectrum of a Po-Be source is from zero to 12 MeV. The mean spectral energy is 4·8 MeV. The neutron yield is, according to published data, from $0{\cdot}95\times10^6$ to $2{\cdot}8\times10^6$ neutr/sec/c of polonium. Point sources of low activity are usually employed for the various dosimetric investigations. Paper [22] describes the design of an emitter for radiobiological purposes consisting of nine sources with a total activity of 200 c arranged on the circumference of a cylinder. A cross-section of the apparatus is given in Fig. 7. The material for protecting the operators is a mixture of paraffin and borax. The arrangement has three chambers. The central chamber is intended for irradiation with fast neutrons and the lateral ones with mixed fluxes of fast and slow neutrons. The flux of fast neutrons in the centra chamber according to the author amounts from a charge of 200 c to about $7{\cdot}6\times10^4$ neutr/cm²/sec. The background of γ-radiation is low and is 0·35 r/hr [22]. In the lateral chambers the fast neutron flux is $6\times10^3$ neutr/cm²/sec, for the thermal neutrons $1{\cdot}2\times10^4$ neutr/cm²/sec and γ-radiation 0·09 r/hr. An advantage of such an emitter is a more even dose field within the object than on unilateral irradiation.

A defect of Po-Be sources is their comparatively short life since the intensity of neutron emission falls off in proportion to the decay in activity of polonium, i.e. in a half-period of 139 days.

Use of various accelerators considerably extends the scope of radiobiological research with fast neutrons as the total neutron yield from the target may reach a value of $10^{12}$–$10^{13}$ neutr/sec. The neutron yield, the energies of the neutrons and the spectrum depend largely on the energy of the accelerated particles, on the type of nuclear reaction utilized to obtain the neutrons and on the thickness of the target in which the neutrons are generated. Without going into a detailed description of the many types of nuclear reactions used to obtain neutrons of different energies and the numerous types of accelerators,

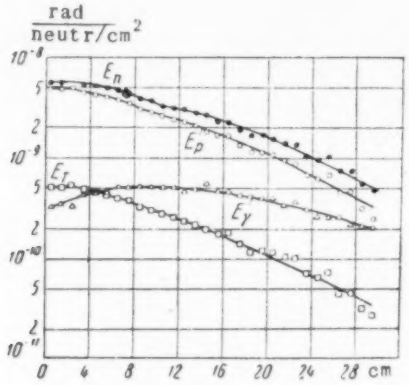


FIG. 5. Distribution of tissue dose components for neutrons with energy 5 MeV. ($E_p$) recoil protons; ($E_\tau$) heavy recoil nuclei; ($E_\gamma$) H ($n$, $\gamma$) D capture; ($E_n$) total dose.

we shall give as an example the dosimetric characeritstics of neutron beams generated in cyclotron. The usual target is beryllium and the accelerated particles protons or deuterons. The neutron spectrum obtained with thick targets is of a complex nature. For example, the energy spectrum of neutrons formed on bombarding a thick beryllium target with deuterons with an energy of 13 MeV reaches 15 MeV. The mean spectral energy is 5·6 MeV. With increase in the energy of the deuterons to 24 MeV, the maximum neutron energy rises to ~ 25 MeV.

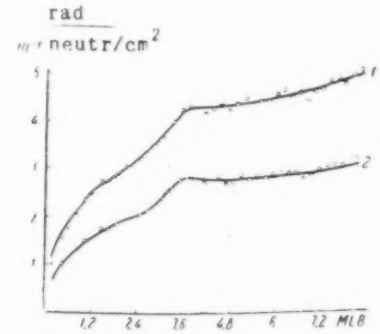


FIG. 6. Relation between first-collision dose and neutron energy. (1) muscle tissue; (2) bone tissue.

On bombardment of beryllium with deuterons (or protons) γ-radiation also develops in the target. The intensity of the γ-radiation may be fairly high, the more so since to the direct flux of γ-rays from the target is added the γ-radiation occurring when the neutrons impinge on the protective devices, etc.

In order to create purer experimental conditions, irradiation is carried out in a special lead housing with a wall thickness of several centimetres [15, 17, 18]. The background of γ-radiation is reduced to such an extent that, depending on the shielding conditions, the tissue dose from γ-radiation is from 5 to 15 per cent of the neutron tissue dose. The use of additional lead protection not only reduces the intensity of the neutron beam but fundamentally alters its spectrum. Hence, a pre-requisite for radiobiological work is to establish the form of the spectrum in actual experimental conditions. The intensity of the neutron tissue dose at a distance of 1 m from the target may reach, in relation to the energy of the accelerated particle, values of several thousands of rads per hour. For example, according to [19], at a distance of 75 cm from the target (without extra lead protection) for deuteron energies of 20 and 24 MeV and a target current of 60 μA the tissue doses are 8400 and 15,000 rads/hr respectively. According to [17], for a deuteron energy of 21 MeV in a lead housing with a thickness of frontal wall of 7·5 cm Pb then on conversion to a distance of 1 m and a current of 60 μA, the intensity of the tissue dose is about 2500 rads/hr.

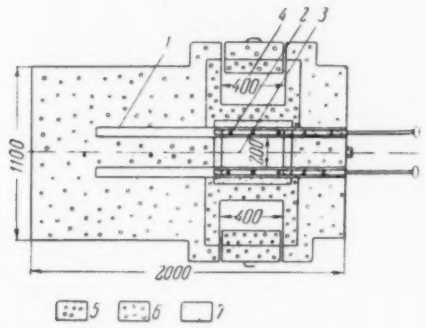


FIG. 7. Cross-section of emitter with Po-Be sources. (1) sources; (2) cassette with sources; (3) central chamber; (4) lateral chambers; (5) mixture of paraffin and borax; (6) paraffin; (7) borax.

On bombarding the target with high-energy deuterons (~ 20 MeV) the neutron flux is in the main directed frontally with maximum intensity along the axis of the beam. Thus, for example, according to [19] at an angle of 16° from the axis of the beam and with a deuteron energy of 24 MeV, the intensity of the flux is reduced by half (at a distance of 1 m from the target and 25 cm from the axis of the beam the flux is reduced by half that on the axis).

High fluxes of fast and thermal neutrons can be obtained in reactors. The neutron flux in the active reactor zone reaches $10^{13}$–$10^{14}$ neutr/cm²/sec. The neutrons obtained as a result of fission on colliding with the substances of the moderator lose their energy and attain thermal velocities. Thus, the neutron spectrum in the active zone is of a more complex character than that at fission. The active zone is also a very powerful source of γ-rays obtained as a result of fission of U or Pu, radioactive decay of the debris and as a result of the interaction of the neutrons and the constructional elements of the reactor. The γ-ray spectrum is very complex and ranges from low energies to those reaching several MeV [23]. Accordingly, use of neutron fluxes directly from the active zone without special filtering devices is not possible.

In order to obtain fluxes of thermal or fast neutrons, resort is usually made to the so-called hot graphite column. Figure 8 schematically depicts, in cross-section, a hot column with a chamber for irradiation as described in paper [24]. The neutrons from the active zone are slowed down in the thick graphite layer reaching an energy corresponding to that of the thermal vibrations of carbon atoms in a graphite lattice. For the absorption of γ-radiation passing from the active zone a layer of bismuth, 22 cm thick, is inserted. Since on capture of thermal neutrons by carbon atoms, hard γ-radiation is emitted, the irradiation chamber has extra bismuth protection 2·5 cm thick. For a reactor power of

26 MW the flux of thermal neutrons in the irradiation chamber is equal to $9\times10^8$ neutr/cm$^2$/sec. To obtain fast neutrons a $^{235}$U convertor is used in the form of a disk 17 cm in diameter and 0·1 cm thick. The thermal neutrons are almost completely (96 per cent) absorbed in the uranium disk causing fission of the uranium atoms. As a result, in the irradiation chamber we have a neutron flux of fission neutrons with a mean energy of about 1·5 MeV. In the arrangement described, the flux of fast neutrons reached a value of $2{\cdot}3\times10^8$ neutr/cm$^2$/sec, i.e. the tissue dose was about 1500–2000 rad/hr. Similar devices have been described in papers [8] and [25]. In paper [9] the flux of thermal neutrons was $1{\cdot}8\times\times10^{10}$ neutr/cm$^2$/sec and the tissue dose of fast neutrons from the uranium convertor was higher.

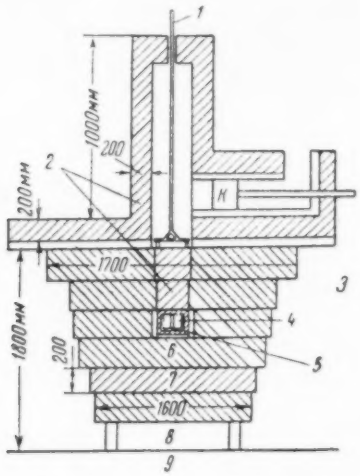


FIG. 8. Schematic cross-section of hot reactor column. (1) insertor rod; (2) iron shielding; (3) reactor shield; (4) irradiation chamber; (5) bismuth shield; (6) graphite; (7) bismuth; (8) ventilation channel; (9) reactor grid.

Despite the fact that use of reactors in a number of cases gives a higher intensity of absorbed dose, the conditions for irradiation with uranium convertors are worse than with use of cyclotrons. In Table 2 taken from the data in paper [24] we give the spectral composition of the neutron flux with use of convertor.

As Table 2 shows, the slow-neutron component accounts for 66 per cent of the total neutron flux, which in relation to the size of the object may make an appreciable contribution to the tissue dose of the fast neutrons. The second drawback is the high non-uniformity of the dose field when using a convertor. According to [24] on changing the distance from the surface of the convertor from 2·5 to 10 cm the neutron flux changes by a factor of 4.

TABLE 2. COMPOSITION OF NEUTRON SPECTRUM

| Energy range | Flux ($\times10^8$ neutr/cm$^2$/sec) | Percentage of total flux |
|---|---|---|
| 0–0·5 eV | 4·16 | 60·5 |
| 0·5–1000 eV | 0·4 | 5·8 |
| 0·1–1·5 MeV | 1·27 | 18·5 |
| 1·5–2·5 MeV | 0·87 | 12·7 |
| 2·6 MeV | 0·16 | 2·3 |

The tissue dose from γ-radiation is also considerable and represents about 25 per cent of the tissue dose of the fast neutrons. Hence, from the point of view of "purity" of radiobiological experiment, i.e. the possibility of obtaining high-energy neutrons, the accelerating devices are more advantageous than reactor beams. On the other hand, the stablility of the output of the reactor, the possibility of using high fluxes of thermal neutrons and also the real possibilities for further improvement in the purity of the beam of fast neutrons create conditions for the further development of radiobiological experiments with fluxes of fast neutrons obtained from reactors.

We have deliberately refrained from an analysis of work with neutron energies into the tens and hundreds of MeV. The phenomena observed on interaction between neutrons of ultra-high energies and the tissue are far more complex. The process of elastic scatter is now joined by processes of deep fission characterized by expulsion from the carbon, nitrogen and oxygen nuclei of secondary protons, deuterons, tritons and α-particles. Individual papers have dealt with investigations of the dosimetric characteristics of ultra-fast neutrons [5].

## SUMMARY

VOL. 5 1960

The material presented suggests that in each particular case of using neutron fluxes, very extensive dosimetric measurements should be made. By dosimetric measurements we mean the whole variety of experimental work and calculation (very laborious) to determine the absorbed dose at different points of the irradiated object for the various ionizing components.

As a rule, use is made of the whole repertoire of methods of measuring ionizing radiations: threshold detectors, ionization, photographic and chemical methods of dosimetry.

Such investigations are an integral part of any radiobiological experiment involving determination of the relative biological effictiveness of neutron fluxes. The terms of reference of the present article do not permit a detailed analysis of the accuracy with which the tissue dose for neutrons can now be defined. This accuracy depends on a number of factors, namely, size of object, neutron energy spectrum, level of background γ-radiation, etc.

It is clear that the greatest accuracy in determination of the absorbed dose can be attained for monoenergetic neutron fluxes with a very low background of γ-radiation.

To obtain such monoenergetic neutrons, use can be made of the nuclear reactions: D $(d, n)$ $^3$He and T $(d, n)$ $^4$He in which neutrons are obtained by bombarding deuterium or tritium with deuterons.

The first reaction is used as a source of monoenergetic neutrons with energy of 2–10 MeV (using a thin target); the second serves to obtain monoenergetic neutrons with an energy of ~ 13 MeV.

Unfortunately, the neutron yield is much smaller than from deuterons accelerated to high (~ 20 MeV) energies and bombarding a thick beryllium target. This fact greatly limits the use of monoenergetic neutrons in radiobiological experiments. With use, for obtaining neutrons, of deuterons accelerated to high energies (for example, in the cyclotron from the $^9$Be $(n, d)$ $^{10}$B reaction) the main flux consists of high-energy neutrons. To find the distribution of neutrons from the deuleron energies in this case offers no difficulties (except the time involved) and the "one-hit" dose can be defined with sufficient accuracy for radiobiological research. Some indication of the accuracy in defining the tissue dose may be afforded by the fact that the first-collision doses computed by two completely different and independent methods (method of threshold detectors and ionization method) differ by 10–15 per cent.

The worst experimental conditions, as we saw above, apply when using fission neutrons (reactors). The presence in the neutron spectrum of a large number of neutrons of intermediate energies and fairly considerable γ-radiation reduces the accuracy in defining the absorbed dose. Break-down of the tissue doses produced by the neutron flux and γ-radiation can hardly be made with an accuracy greater than 15–20 per cent. These same causes, i.e. the need for break-down of the ionizing components, reduce the accuracy in determining tissue doses in objects with a large scattering mass when together with absorption of the primary beam of neutrons the role of the various components in producing the ionization changes.

It is obvious that these remarks do not apply to experiments with neutrons in which only relative dosimetry is required, i.e. with retention of constant systems of irradiation. This is true, for example, in the study of the effect of various factors on radiosensitivity; study of the oxygen effect, dose rate, etc. In these cases, the constancy of the irradiation conditions may be ensured with an accuracy greatly exceeding the requirements of modern radiobiology.

Expressing the dose absorbed in rads or ergs implies determination of the total energy of the ionizing radiation absorbed by the irradiated substance per unit mass. Calculation of the absorbed dose (from the processes of ionization) is only the first step in dosimetry.

The second step is to define the absorbed dose in relation to the linear density of the absorbed energy (l.d.e.), i.e. in relation to the factor which determines the difference in the r.b.e. values for different forms of ionizing radiations. This aspect of dosimetry is still in the initial stages of development with regard to neutrons and only isolated papers on it have appear d [26–29]. Moreover, in an analysis of the biological effect of heavy recoil nuclei we are convinced of the need to work out experimental methods to determine the losses of the particle energy connected with such processes as excitation of the biological tissue molecules. The development of this work will bring closer an understanding of the primary mechanism of the energy exchange of the ionizing particle by qualitatively evaluating the role of the particular processes responsible for the biological effect of ionizing radiations.

In conclusion, the author regards it as his pleasant duty to express his thanks to Iu. S. Bregadze, V. A. Kvasov, Ye. A. Liberman and Ia. L. Shekhtman for valuable advice.

*Translated by* A. CROZY

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# A METHOD FOR STUDYING HIGH-FREQUENCY POTENTIALS OF THE ELECTROENCEPHALOGRAM *

V. A. IL'IANOK

Institute of Higher Nervous Activity, U.S.S.R. Academy of Sciences, Moscow; Faculty of Fundamental Biology of Lomonosov State University, Moscow

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As is known, the electroencephalogram (e.e.g.) is a complex curve, the shape of which is determined by the simultaneous presence of biopotentials of different frequency and amplitude. The amplitudes of all e.e.g. frequencies are not stable; they fluctuate constantly around a certain mean level, which explains the continuous variation in the visible e.e.g.

On visual analysis of the e.e.g., account is taken only of a small part of the information it contains since interpretation of the high-frequency components from the record of the e.e.g. itself is extremely laborious and possible only with rapid scanning within short time intervals. In such interpretation, a number of Soviet electrophysiologists [1 2] have shown that there are in the e.e.g. also high-frequency potentials — up to several hundreds of cycles per second.

It should be noted that the generally-accepted evaluation of the e.e.g. amplitudes, calibrated in microvolts, does not correspond to the true amplitude of the given frequency because the potential recorded in the visible e.e.g. is in fact the sum of the amplitudes of a number of frequencies [3]. Therefore, accurate quantitative assessment of the potentials of the individual frequencies is practically impossible on visual analysis of the e.e.g.

Automatic e.e.g. analysers of the "Kaiser", "Alvar" and "Ediswan" types, etc permit much more precise quantitative analysis of the biopotentials of the individual frequencies but they can only separate out low frequencies — up to 30 c/s.

Our proposed instrument, a Grey Walter harmonic analyser manufactured by Messrs. Ediswan firm but somewhat modified, can give objective quantitative evaluation of the averaged amplitudes both of low and high frequencies — up to several hundreds c/s.

As is known, the Grey Walter analyser consists of 24 resonance amplifiers (filters) each of which passes only one frequency from 1·5 to 30 c/s. The frequency separated by the filter is integrated and enters a condenser charging it within 10 sec. A special contact device alternatily removes the accumulated charges from the condensers and feeds them into the amplifier. The latter is connected to the pen of the analyser which traces a series of peaks on the tape of the ink-writing oscillograph. The height of each peak reflects the energy of the given frequency averaged over a 10 sec period of analysis. At the output of each filter there are three reservoir capacitors — known as A, B and C groups. While the group A capacitors are serving the function of storage, the earlier accumulated charges are being removed from the group B capacitors. The capacitors then exchange their functions. This ensures continuous

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output of the e.e.g. frequency spectrum every 10 sec. The capacitors of group C can be supplied with 1/3rd, 1/5th, 1/7th, 1/9th or 1/11th part of the energy separated by each filter. This permits recording not only of the averaged spectrum for each 10 sec, but also the averaged values of the separated frequencies for every 30, 50, 70, 90 or 110 sec as desired. Such averaging is very convenient in a number of cases since it levels out random variations in the e.e.g.

In redesigning the Grey Walter analyser, the phase-shifting RC elements were changed for certain filters after which it was possible to tune these filters to higher frequencies. The parameters of the phase-shifting RC elements were found experimentally for each of the separated frequencies. After redesigning, all the filters were located in sequence of rising amplitude.

In selecting the frequencies for separation, we started from the following premises: (1) the low and fundamental α-frequencies must be separated by a larger number of filters than the high frequencies; (2) as far as possible for the remaining low frequency filters, filters must be prepared separating the low and higher frequencies — harmonics; and (3) the filters cannot be used for 50 and 100 c/s since they react strongly to induction from an a.c. network even after the most careful elmination. With this rn mind, we redesigned the analyser in such a way that it started to separate the following 24 frequencies from the e.e.g. — 1·5, 3, 4, 5, 6, 9, 10, 11, 12, 15, 20, 24, 30, 40, 48, 60, 72, 80, 96, 120, 160, 240, 320 and 480 c/s (Fig. 1).

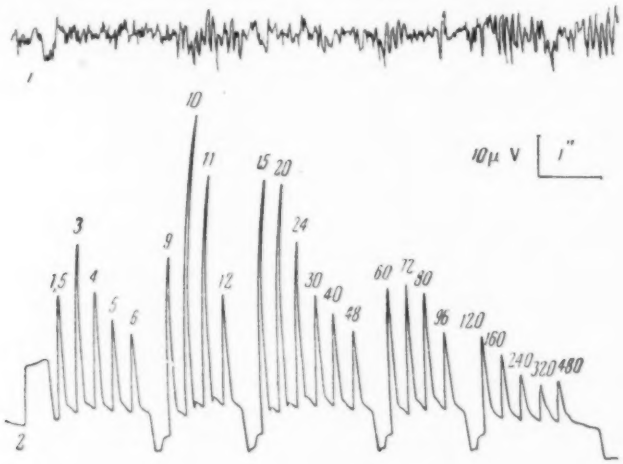


FIG. 1. E.e.g. of occipital region of human brain with unipolar recording. (1) e.e.g. record; (2) amplitudes of individual e.e.g. frequencies given by redesigned Grey Walter analyser and averaged over 50 sec.

The analyser filters up to a frequency of 96 c/s were tuned by means of a very stable, low-frequency NGPK-2 generator and for higher frequencies — above 100 c/s — with ZG-10 audio-oscillator. As the frequency of audio-oscillators is unstable and the frequency scale only roughly corresponds to the frequency generated, high-frequency filters should be tuned with simultaneous use of both generators. The frequency standard, a multiple of the desired frequency is taken from the low-frequency generator connected to the vertical deflector plates of the tube of the cathode oscillograph. The frequency of the audio-oscillator is fed to the horizontal plates of the tube. The desired high frequencies are then accurately set by means of Lissajous figures (4).

On tuning the filters, the amplitude of the signal from the generator fed to their input is measured from the deviation of the beam of the tube. For accurate measurements of the amplitudes of different frequencies, a signal by-passing the amplifier is delivered directly to the vertical plates of the oscillo-

graph. The transient capacitances between the input and the plates of the oscillograph must be not less than $\pm 0{\cdot}5$ μF.

The sharpness of the filter tuning is of fundamental importance for the accuracy of analysis of the individual e.e.g. frequencies. The instructions on the analyser recommend fairly wide tuning of the filters so that a deviation of signal of $\pm 1$ c/s from the filter frequency will give 44 per cent of the peak amplitude. We consider that all the filters must be tuned much more sharply, since on wide tuning the total energy of all frequencies passed by a given filter will not reflect the value of the potentials of this frequency. The reason for this is that with a small increase in amplitude of the fundamental separated frequency and considerable fall in the amplitude of the adjacent frequencies (or vice versa)

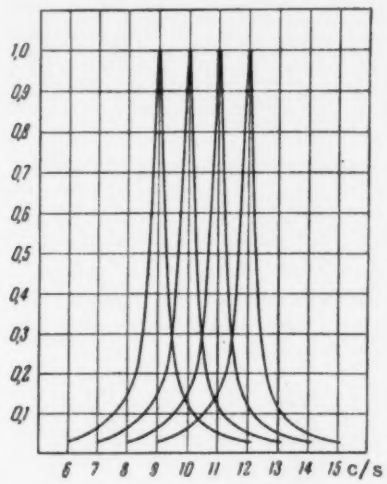


FIG. 2. Characteristics of transmission of filters separating from the e.e.g. the α-frequencies of 9, 10, 11 and 12 c/s.

the indications of the filter may not change at all or change very little and will not always be synonymous with changes in the fundamental frequency to be isolated. For each filter, to separate from the e.e.g. essentially one frequency and as few neighbouring ones as possible, we tune all the filters separating potentials with a frequency up to 96 c/s very sharply (as indicated in Fig. 2): deviation of the frequency of the signal by $\pm 1$ c/s from the filter frequency gives only one-eighth peak amplitude. Higher frequency filters are tuned somewhat wider — deviation of signal by $\pm 1$ c/s from filter frequency gives about one-fifth peak amplitude.

As is known, the amplitudes of high-frequency potentials are tens of times less than those of low frequencies. Further, the amplifiers of the biocurrents have a steep slope in the high frequency region. For the simultaneous recording of all the frequencies to be separated it is necessary that the high-frequency filters have large amplification. It was found convenient in practice for filters from 15 to 48 c/s to have double, the 60–96 c/s filters fourfold and for 120–480 c/s, 8 times the amplification of the 1·5–12 c/s filters (Fig. 3).

At such amplification, high-frequency filters start to respond to slow waves of high amplitude. Therefore, the biocurrents are supplied to the second half of the resonance amplifiers of the analyser, separating the potentials with a frequency of from 30 to 480 c/s, via a special RC-filter which does not pass the mean frequency of the α-rhythm at all (10·5 c/s) and very substantially reduces the neighbouring and low frequencies (Fig. 4 and [5]).

It should be remarked that the band-pass width and amplification of each filter gradually change. Such changes are more often observed in high-frequency filters. Therefore, a regular check and frequency trim of all the analyser filters is necessary.

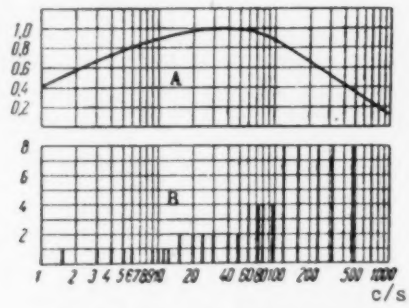


FIG. 3. (A) Frequency characteristic of biocurrent amplifier for a time cnonstant of 0·03 and a filter of upper limit 75 c/s; (B) comparative amplifications of analyser filtres.

Using such a redesigned analyser and allowing for the different amplification of the filters and frequency characteristic of the encephalograph, one can construct e.e.g. frequency spectra which reflect the values of the amplitudes of the separated frequencies averaged over a time lapse both in normal conditions and on change due to various factors (Fig. 5).

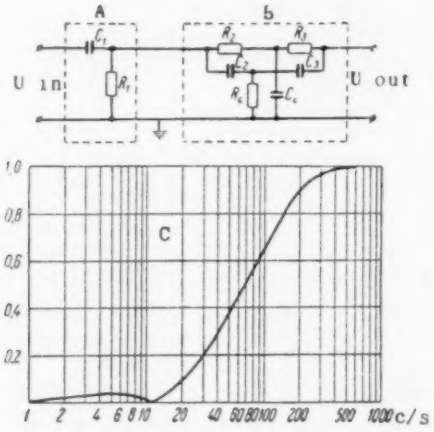


FIG. 4. Combined RC filter and its frequency characteristic. (A) filter separating low frequenciese (B) band elimination filter not passing mean frequencies; (C) overall characteristic of passage of thes; filters. $R_1 = 11$ k$\Omega$, $R_2 = 360$ k$\Omega$; $R_3 = 47$ k$\Omega$; $R_4 = 30$ k$\Omega$; $C_1 = 1$ $\mu$F; $C_2$, $C_3$, $C_4 = 0{\cdot}15$ $\mu$F.

In order to evaluate the true value of the averaged amplitudes of the individual e.e.g. frequencies the indicators of the analyser are calibrated in microvolts by supplying sinusoidal oscillations to their input. The use of such units for assessing the indications of the analyser is to a certain extent arbitrary as the value of the indications of each filter reflects the averaged energy of the given frequency and consequently depends not only on the amplitude of the signal but also on its duration, i.e. on the "electrical area" of the signal, the shape of which is not exactly sinusoidal [6].

While noting the known inaccuracy of such units, we nevertheless consider it possible to use them since they not only allow comparison of the amplitude of the individual frequencies among themselves, but also comparison of the frequency spectra for different subjects obtained at different times with non-uniform amplification of the analyser and encephalograph.

The averaged value of the amplitude of the individual frequency thus measured, for example, the dominating frequency of the α-rhythm, is much less than the total amplitude of all frequencies of the α-rhythm seen on visual analysis from the calibrated pulses of the encephalograph. By means of such an analyser it is convenient to study reproduction by the brain of the rhythms of flashes of light over a wide frequency range.

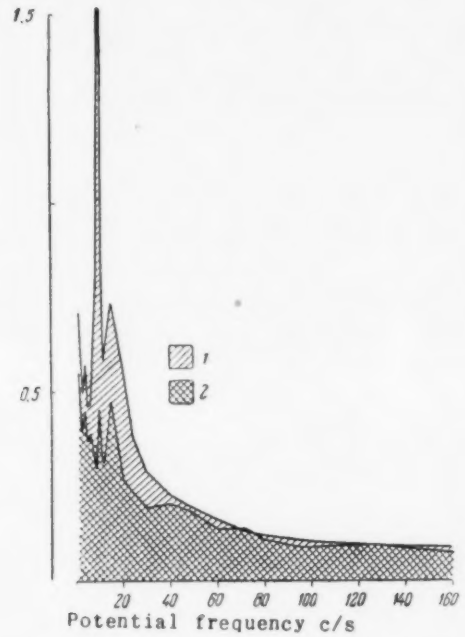


FIG. 5. Averaged frequency spectrum of e.e.g. of occipital region of human brain. (1) in darkness; (2) on illumination of eyes with non-flickering light. Reduction in amplitudes of low frequencies is visible and is particularly great in the region of the α-rhythms. Ordinates, amplitude of e.e.g. in mV.

Usually, to obtain flashing light, Soviet or foreign ("Alvar", "Kaiser" etc) photostimulators are used with a pulsed lamp. Such stimulators, in our view, have the following fundamental defects: (1) each light flash is accompanied by an audible click; (2) in many photostimulators with a pulsed lamp, increase in the frequency of the light pulses is accompanied by decrease in their brightness; (3) the upper limiting frequency of the flashes is not more than 50–70 per sec; and (4) the maximal intensity cannot be obtained for high frequency flashes, which is of great importance for assimilation of high rhythms. These and a number of other shortcomings limit the possibilities of a wide use of such photostimulators for scientific purposes.

We propose a simple mechanical photostimulator largely free of these defects. The layout of the stimulator is shown in Fig. 6. It consists of a synchronous motor 1, which via a system of reduction gears, 2–3 rotates disk 4. This disk sets in motion a small wheel with rubber rim 5 which drives a sector disk 6, with five or ten notches, located on its axis. Wheel 5 by means of special rack can move along its axis to touch disk 4 at any point between its centre and periphery. This ensures the necessary speed

of rotation of sector disk 6. The illuminating system includes a 500 W cine-lamp 7, condensor 8, objective 9 and a horizontal slit diaphragm 10.

Such a design of stimulator gives: (a) light flashes with a frequency from 3 to 120 per sec for a sector disk with five notches or from 6 to 240 per sec for a sector disk with ten notches; (b) smooth change in frequency within these limits; (c) high stability of the set frequency; (d) a practically square form of the light stimuli — because of the small aperture of the objective (1 cm²) and relatively large radius of the sector disk (23 cm); (e) possibility of changing by means of other sector disks the ratio of the duration of light and darkness; (f) high brightness of the light flashes; and (g) change in brightness within wide limits without change in form and spectral composition of the light pulses.

Together with the use of the slit diaphragm, the intensity of the flashes may be varied stepwise by means of sets of frosted glass filters with transmission coefficients of 1/5, 1/25, 1/125 and 1/625 which are inserted into grooves 11.

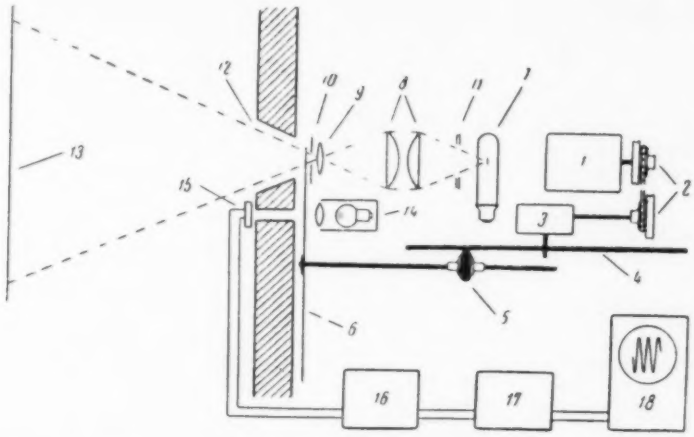


FIG. 6. Schematic diagram of photostimulator and apparatus for obtaining precise correspondence of frequency of light flashes and peak frequency of analyser filters.

The light in the form of a circle of 74 cm diameter impinges, via an aperture in the wall of the chamber 12, on the white screen 13 and is seen by the subject at an angle of 95°. The light reflected by the screen is measured at the level of the eyes of the subject by a highly sensitive luxmeter consisting of an antimony-caesium photocell and a photocurrent amplifier assembled in the bridge circuit on an IE1P electrometer valve [7].

Because of the sharp tuning of the filters of the analyser, the slightest divergence betweeen the frequencies of the filter and stimulus gives an apparent strongly reduced effect of assimilation of the rhythm of the flashes of light. Therefore, the stimulator has another illuminator 14, on turning on this lamp the flickering light falls on a selenium photocell 15 connected to one of the channels of the biocurrent amplifier 16 and through it to analyser 17. One of the analyser filters is linked to the oscillograph 18. The flash frequency is changed until it accurately coincides with the frequency to which the given filter is tuned: this is indicated by the maximum amplitude of the variation of the trace on the oscillograph tube. The agreement of the filter frequency and stimulus is checked regularly throughout the experiment and this ensures accuracy of analysis.

The results of our experiments [8] show that use of the modified harmonic analyser in association with the mechanical photostimulator gives a high degree of accuracy in investigations of changes in the e.e.g. frequency spectrum under the influence of rhythmic light. Thanks to the sharply tuned filters, the analyser clearly demonstrates even slight changes in the electrical activity of the brain due to rhythmic

light stimulation. For example, we established assimilation by the human cerebral cortex of the rhythm of light flashes with frequencies of 5–40 per sec on illumination of the eyes of the subject at a level of only 0·08 lx. For high luminance assimilation of the rhythm not only of low frequencies of flashes but of those such as 60, 72 and 80 and in some subjects even 96, 120 and 160 per sec was observed. Such reactions of the brain were not known previously since the minor changes in the small amplitudes of high-frequency potentials are difficult to detect with the most careful visual inspection of the e.e.g.

By the use of the arrangement described, we together with Samsonova [9], have been able to demonstrate another phenomenon expressed in the ability of the human brain to assimilate not only the rhythm of one light stimulus but also the different, non-uniform rhythms of two or three light stimuli given simultaneously on a white screen in front of the subject and to raise the amplitude of their sum and different frequencies.

Hence, the suggested method gives assistance in arriving at an objective assessment of the averaged amplitudes of the individual frequencies of the total electrical activity of the brain. Investigation not only of the slow but of the fast components of the e.e.g. is of immense importance since it extends the possibility of using electroencephalography to study the physiological laws of functioning of the brain.

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VOL. 5 1960

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* There is a translation into English of this work.

# PRINCIPLE OF PUPIL SCANNING*

A. R. SHAKHNOVICH

Burdenko Institute of Neurosurgery, U.S.S.R. Academy of Medical Sciences, Moscow

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IN order to record the reflexes of the pupil, dynamic measurement must be made of its area or diameter.

There are now various photoelectrical procedures available for measuring the area of the pupil [1, 2]. All these methods are based on measurement of the number of infra-red rays reflected from

* Biofizika **5:** No. 4, 493–496, 1960.

the iris. Change in the size of pupil leads to change in the number of these rays, reflected in the electrical potentials formed at the photocell. However, the number of these rays varies not only with changes in the area of the pupil but also with the constant movements of the eye and eyelid and other secondary factors. The imprecision of this method was demonstrated by the controlled experiments of Löwenfeld [3].

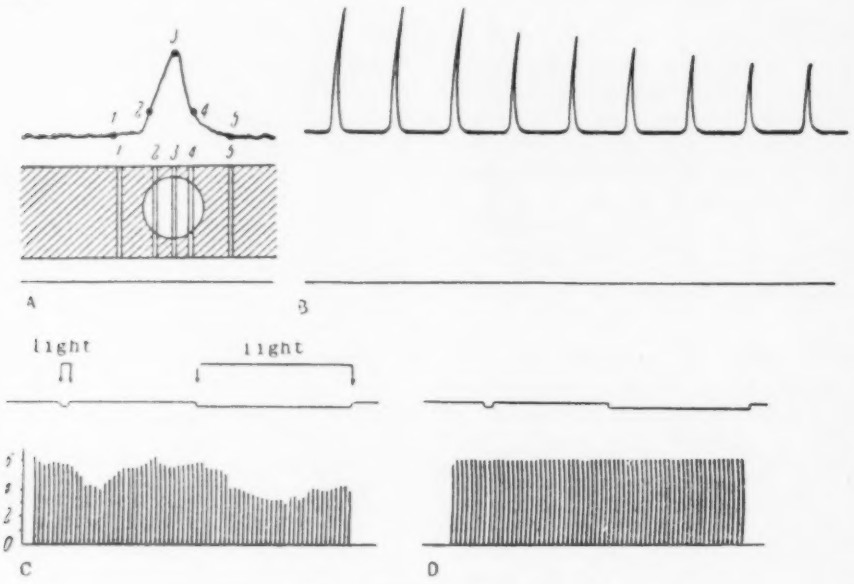


FIG. 1. Explanation in text.

For the dynamic measurement of the diameter of the pupil the Belliarminov method [4] is applied. However, as has already been shown by us, the constant movements of the eye exclude sufficiently reliable results when the method is applied in man.

The most refined method for recording pupillary reflexes is now cinematography. The diameters of the pupils may be quite accurately measured on ciné-frames and the result obtained is independent of the movements of the head, eyes and eyelids or other secondary factors. However, the long and laborious process of measuring the pupil diameters on ciné-frames has considerably restricted the application of this method. Thus, while ciné-recording of the pupillary reflexes is made in 1 min, at a rate of 10 frames/sec, it takes several hours to plot the curves of the reactions of both pupils. It is understandable that such a technique can only have limited use in physiological experiments and is entirely unsuitable for wide clinical use.

In an earlier paper [5] we demonstrated the possibility of automatic scanning of the pupil for determining its diameter. The image of the pupil is moved perpendicular to the slit diaphragm behind which is placed the photographic film. In these conditions the diameter of the pupil is printed on the film in a column form.

We have now applied such a scanning technique using not only photographic, but also electronic methods. The image of the pupil moves in a direction perpendicular to the photocell, the working surface of which has a narrow rectangular shape. Figure 1 (A) depicts the successive positions of the photocell in relation to the image of the pupil on the ciné-frame. As can be seen from the figure, the area of the photocell illuminated during movement of the pupil changes. The electrical current formed in the photocell is amplified and its changes are recorded on the pen-recorder. At the moment when the diameter of the pupil crosses the photocell the pen reaches its maximum (position 3 in Fig. 1 (A)).

Thus, the size of the maximum rise of the pen accurately corresponds to the diameter of the pupil. In Fig. 1 (B) is shown an ink record of the pulses formed in the photocell when the images of the pupils of various dimensions, as recorded with consecutive ciné-frames, cross it. The speed of movement of the paper was less than in the preceding record and in the intervals between the frames crossing the photocell the light was automatically cut out. As Fig. 1 shows, reduction in the size of the pupil in

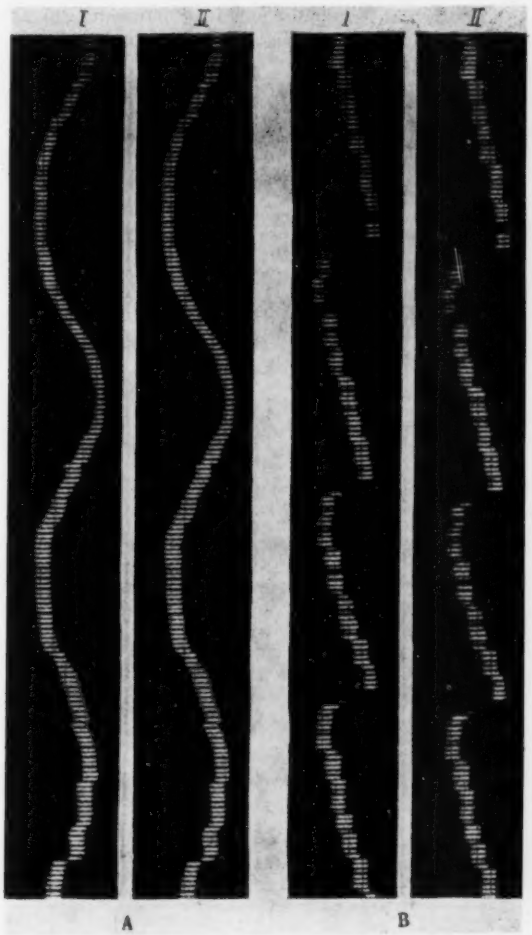


FIG. 2. (A) tracking of swinging pendulum; (B) movement of eyes on reading four short lines. (I) left eye; (II) right eye.

the ciné-frames leads to reduction in the pulses in the photocell, i.e. we have amplitude modulation of the signal. The effect of a further reduction in the speed of the paper (Fig. 1 (C)) is that the pen of the recorder traces out the diameter of the pupil on each ciné-frame in the form of a column. This ensures the complete linear dependence of the size of these columns on the diameters of the pupils on the ciné-frames. The size of the first column is made to agree, by a definite scale, with the diameter of the pupil on the ciné-frame by varying the illumination; from the scale at the origin of the curve the absolute dimension of the pupil diameter can be established in millimetres. In order to demonstrate

the accuracy of our records special controlled investigations were made. Atropine and pilocarpine were instilled into the subject's eye to produce complete paralysis of the iris muscles and immobility of the pupil. We then obtained on consecutive ciné-frames images of the pupils of completely uniform size. The record of the pupil diameters with such a film gave columns of identical size (Fig. 1 (D)). A major advantage of electronic over photographic scanning is the possibility of obtaining a record much more rapidly without additional development of the film. This is particularly important for mass clinical investigations. However, the method of photographic scanning also has its positive aspects.

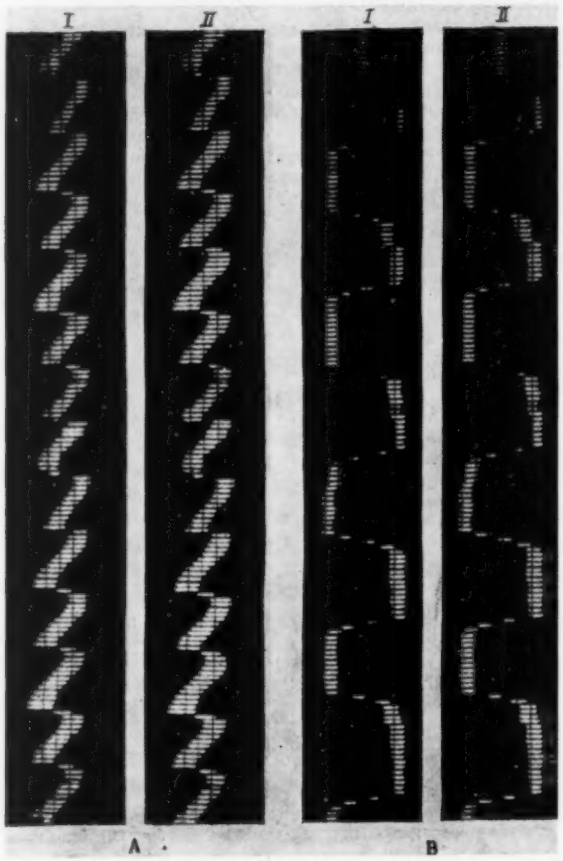


FIG. 3. (A) Optokinetic nystagmus; (B) alternate inspection of two points at angle of 25° horizontally. (I) movement of left; (II) moment of right eye.

Unlike the electronic method, on photographic scanning the curves obtained show not only of the responses of the pupil but also movements of the eyes. As was shown by us earlier [3] this is particularly important for study of the relation between the responses of the pupils and the movements of the eyeballs on convergence. Moreover, application of such a method makes it possible to record the eye movements not only on convergence but also on reading, optokinetic nystagmus, tracking of objects, etc. (Fig. 2, 3). Such records do not require the fixing of a mirror or any marking on the eyeball and allows rapid and easy recording of the movements of the eyes in the usual clinical condi-

tions. Hence, combination of electronic and photographic scanning of the pupil with ciné-frames permits wide use of the cinematographic method for recording both pupillary reflexes and movements of the eyeballs.

*Translated by* A. CROZY

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* There is a translation into English of this work.

VOL. 5 1960

# AUTOMATIC DEVICE FOR SWITCHING ELECTRICAL INSTALLATIONS ON AND OFF*

A. B. BRANDT

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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THE need often arises in an experiment for automatic switching on and off of the most diverse instruments and installations. However, it is not always possible to obtain a suitable device.

For automatic switching on and off when irradiating plants at a definite set time of the day we designed, on the basis of a seven-day clockwork mechanism (a self-recording thermograph or hygrograph) a simple device which is easy to produce.

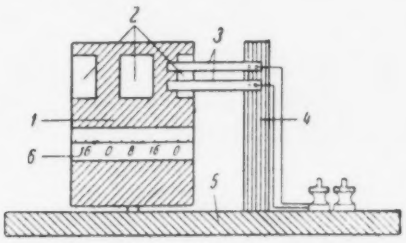


FIG. 1. Diagram of device for automatic switch on and off of electrical installation. (1) rotating drum of thermograph or hygrograph with internal closkwork mechanism; (2) copper contact plates; (3) springy copper brushes; (4) brush holder; (5) base of weekly thermograph, (6) time scale.

The mechanical part of the self-recording thermograph is a drum rotating by means of clockwork On this drum are riveted copper plates of such a length that the time of passage along them of contact

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brushes on rotation of the drum corresponds to the necessary time for switching on of the apparatus. The distance between the two adjacent plates corresponds to the time of rotation of the drum in the period when the apparatus is switched off, since the ebonite casing of the drum is an insulator.

At the level of the plates two springy copper brushes press on to the drum. The instrument is connected in series. At the moment of contact between the two brushes and copper plate, on rotation of the drum, the circuit is switched on; as the plate passes on the circuit is broken. For convenience of observation of the accuracy of the clockwork mechanism, on a part of the drum clear of plates a strip of paper with divisions corresponding to the hours and days of the week is fixed.

A general view of the instrument is given in Fig. 1.

In order to alter the duration of cut-in of the apparatus one of the brushes can be made adjustable and on displacement of one brush relative to the other the time of switch-on of the apparatus is reduced. It should be noted that use of flat plates, as in Fig. 1, is possible only for switching on installations with a power not more than 300 W at a voltage of 120–220 V. For switching on more powerful plant, in order to prevent sparking on making and breaking contact, the plates on the drum should be so arranged that one of their edges lies adjacent to the surface of the body of the drum with the opposite edge raised 2–3 mm. Between the copper plates it is desirable to set up similar shaped plates of insulator (Fig. 2) whereby the momentary making and breaking of the brush contacts is achieved without sparking.

To prevent possible heating of the body of the drum on cutting out the brushes it is advisable to place the contact flat plates not on the body of the drum but on an asbestos-cement collar which is in turn slipped onto the drum. The apparatus described can be used for connecting in a secondary circuit any magnetic relay-starter. The power of the apparatus to be switched on is, in this case, unlimited.

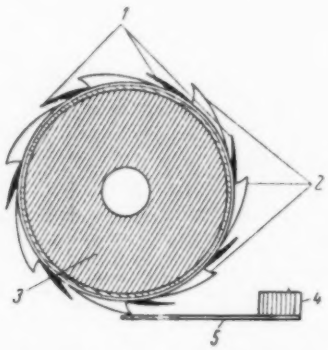


FIG. 2. Design of drum with plates for switch on of high-power apparatus. (1) plates of organic glass (ebonite, textolite); (2) moulded copper contact plates; (3) rotating drum with internal clockwork; (4) brush holders; (5) springy copper brush.

Where necessary, several rows of contact plates with a suitable number of brushes can be arranged on the rotating drum whereby the application of the proposed device is considerably extended.

*Translated by* A. CROZY

# EFFECT OF BROMINE IONS ON THE ACTIVITY OF THE FROG RETINA*

L. M. TSOFINA

Institute of Biological Physics, U.S.S.R. Academy of Sciences Moscow

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VOL. 5 1960

THE effect of bromides on the activity of the central nervous system has been widely studied and it has been observed that bromine enhances the "inhibitory" process. Such work has been done on intact organisms by the conditioned reflex method, a method which cannot reveal the mechanisms of action of bromine.

Investigations with radioactive bromine [1] have shown that the bromine content of different parts of the brain does not change for different states of the central nervous system, this points to the absence of redistribution of bromine between these areas. On injection of labelled bromides, the bromine content increases simultaneously for all sections of the brain in proportion to its original content.

In *in vitro* experiments it has been shown that replacement of the chlorine in the medium bathing the cells by bromine involves a number of changes in the activity of the cells. Thus, on replacing the chlorides of Ringer solution by bromides, there is intensified tension of the muscle fibres on contraction, increased duration of the contraction and a simultaneous increase in the heat production of the muscle [2]. In poorly excitable muscle fibres the threshold of excitation is lowered [3]. Study of the electrical phenomena shows that bromine increases the negative after potential and its duration [4] and causes a 1·5-fold increase in the resistance of the muscle membrane [5].

On immersing the muscle in Ringer solution in which the chlorine has been replaced by bromine, the outflow of chlorine ions from the cells is retarded [4] while that of potassium is apparently increased [6]. It is important to note that in all the papers mentioned the effect of bromine was investigated in comparison with the effect of other anions. It has been noted that in its potency of effect bromine occupies quite a definite position in the lyotropic series of anions, namely, $Cl < Br < NO_3 < I$. There are only very indefinite and occasionally conflicting findings in the literature [7, 8] on the change in the resting potential and threshold of excitation of various muscle and nerve fibres.

In our work we investigated the effect of chlorine, bromine and iodine as well as $NO_3$ ions on the activity of the retina of the frog eye since it is known to contain nerve cells which in structure and origin are very similar to the nerve cells of the brain.

## MATERIALS AND METHODS

The work was performed on the isolated eyes of *Rana radibunda* and *R. temporaria*. The cornea, crystalline lens and vitreous humour were removed from the eye and with oscillographic control and using a binocular lens, the recording microelectrode was attached to the retina. The second electrode was in contact with the outer surface of the eye. The eye was filled with Ringer solution of the following composition: NaCl (or NaBr, NaI or $NaNO_3$) 111 mmole, KCl 1·9 mmole, $CaCl_2$ 1·1 mmole and $NaHCO_3$ 2·4 mmole. The solutions were changed with the aid of a pipette. The retina was stimulated by visible light and the number of nerve impulses recorded from a single ganglion cell were counted on an apparatus of type D. A detailed description of the experimental arrangement is given in paper [9].

* Biofizika **5**: No. 4, 498–501, 1960.

## RESULTS OF EXPERIMENTS

The three experiments in the first series were concerned with the effect of Br ions on the number of nerve impulses on stimulating the frog retina with flashes of light. The number of nerve impulses was counted after each flash for a considerable period of time. After several stimulations the Ringer solution containing NaCl was replaced by the solution containing NaBr and the stimulations repeated. The results of this experimental series are given in Table 1, which shows that bromine increased the number of nerve impulses 2–5 times.

The findings of the second experimental series were obtained by stimulating the retina with rhythmic alternation of light and dark, the frog retina being illuminated for 1 sec followed by 1 sec darkness. After ten such stimulations, i.e. 20 sec, the eye was rested for 30 sec. Following the rest, the stimula. tions were repeated several times — from 3 to 16 — after which the Ringer solution was changed. The results of the six experiments of the second series are given in Table 2 and Fig. 1.

TABLE 1. EFFECT OF BROMINE IONS ON NUMBER OF NERVE IMPULSES ON STIMULATION OF RETINA WITH FLASHES OF LIGHT

| | Ringer solutions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | I | | II | | III | | IV | | V |
| | NaCl | NaBr | NaCl | NaBr | NaCl | NaBr | NaCl | NaBr | NaCl |
| | Experiment No. 1 | | | | | | | | |
| No. of measurements | 15 | 13 | — | — | — | — | — | — | — |
| Scatter of results | 5–12 | 28–37 | — | — | — | — | — | — | — |
| Mean | 10±0/0·019 | 32±0·8 | — | — | — | — | — | — | — |
| | Experiment No. 2 | | | | | | | | |
| No. of measurements | 22 | 10 | 10 | 6 | 7 | 8 | — | — | — |
| Scatter of results | 6–15 | 40–64 | 5–19 | 36–50 | 5–13 | 24–35 | | | |
| Mean | 11±1·0 | 50±2·8 | 11±1·1 | 42±2·0 | 9±1·0 | 28±3·2 | | | |
| | Experiment No. 3 | | | | | | | | |
| No. of measurements | 19 | 16 | 18 | 6 | 18 | 16 | 16 | 8 | 10 |
| Scatter of results | 9–17 | 15–26 | 8–22 | 11–27 | 4–13 | 5–20 | 4–10 | 34–64 | 3–15 |
| Mean | 13±0·6 | 22±0·8 | 17±0·9 | 20±2·9 | 10±0·7 | 11±0·9 | 6±0·4 | 44±3·5 | 8±1·1 |

It will be seen that for this experimental design, replacement of chlorine by bromine ions increased the number of nerve pulses from the retina.

It should be noted that in our experiments bromine exerted its effect almost immediately after changing the solution and that the effect was reversible

On replacing the NaCl of Ringer solution by NaI and $NaNO_3$ the number of nerve impulses from the ganglion cells of the retina increased and to a much higher degree than in the presence of bromine ions. The results of the two experiments of this series are given in Table 3 and 4 and Fig. 2 and 3. Reference must be made to the fact that these experiments were in the main conducted in March when the isolated eyes very rapidly fatigue and die. But even on these "deficient" specimens, the effect of the I and $NO_3$ ions is obvious.

It follows from the experimental findings that replacement of chlorine by bromine in the intercellular fluid exerts a direct influence on the activity of elements of the central nervous system. I and $NO_3$ ions lead to a still greater increase in retinal activity. It may be supposed that the absence of a direct effect of these anions on the central nervous system in *in vivo* experiments is to be explained by the fact that iodine is very rapidly eliminated from the blood via the thyroid and that $NO_3$ ions are not found in the circulating blood. Only bromine remains in the blood in ionic form, possibly leading to its known effects.

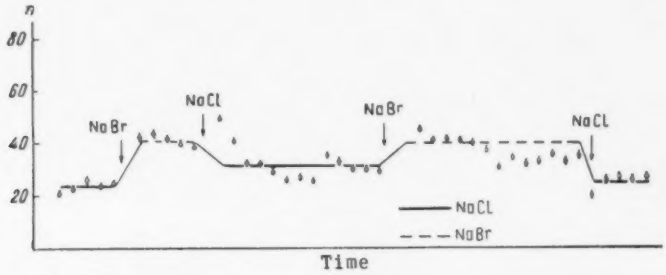


FIG. 1. Effect of Br ions on number of nerve impulses. (Experiment No. 4). Ordinate, number of impulses per sec (*n*).

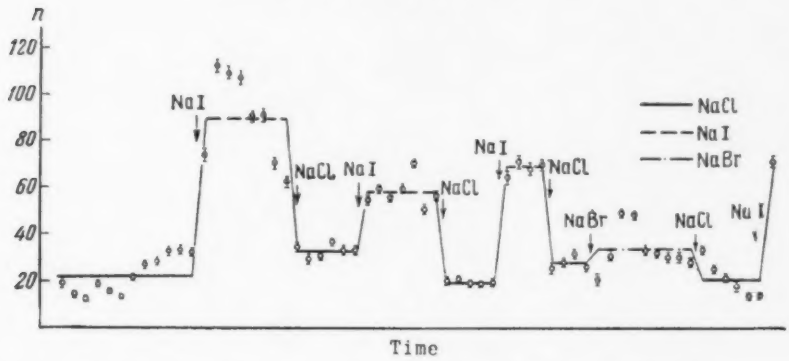


FIG. 2. Effect of I and Br ions on number of nerve impulses (*n*).

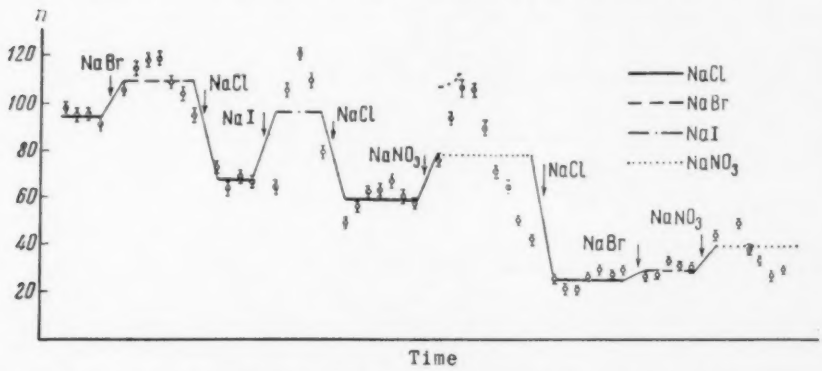


FIG. 3. Effect of Br, I and $NO_3$ ions on number of nerve impulses (*n*).

TABLE 2. EFFECT OF BROMINE IONS ON NUMBER OF NERVE IMPULSES ON STIMULATION OF RETINA WITH ALTERNATION OF LIGHT AND DARK (RHYTHM — 1 SEC)

| | Ringer solutions | | | | |
|---|---|---|---|---|---|
| | I | | II | | III |
| | NaCl | NaBr | NaCl | NaBr | NaCl |
| | Experiment No. 1 | | | | |
| No. of measurements | 30 | 60 | 70 | — | — |
| Mean number of impulses | 42±0 | 54±2·6 | 34±1·4 | — | — |
| | Experiment No. 2 | | | | |
| No. of measurements | 100 | 90 | 130 | — | — |
| Mean number of impulses | 77±2·0 | 102±4·2 | 84±4·1 | — | — |
| | Experiment No. 3 | | | | |
| No. of measurements | 90 | 140 | 100 | — | — |
| Mean number of impulses | 33±1·8 | 41±3·5 | 25±2·5 | — | — |
| | Experiment No. 4 | | | | |
| No. of measurements | 50 | 50 | 130 | 140 | 50 |
| Mean number of impulses | 24±0·1 | 41±3·5 | 32±1·8 | 40±2·7 | 25±1·3 |
| | Experiment No. 5 | | | | |
| No. of measurements | 50 | 100 | 150 | — | — |
| Mean number of impulses | 16±0·1 | 24±1·4 | 24±2·7 | — | — |
| | Experiment No. 6 | | | | |
| No. of measurements | 70 | 160 | 70 | 150 | — |
| Mean number of impulses | 29±1·7 | 62±6·1 | 21±1·7 | 30±1·9 | |

TABLE 3. EFFECT OF IODINE AND BROMINE IONS ON NUMBER OF NERVE IMPULSES ON STIMULATION OF RETINA WITH ALTERNATION OF LIGHT AND DARK (RHYTHM — 1 SEC)

| | Ringer solutions | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | I | | II | | III | | IV | | V | |
| | NaCl | NaI | NaCl | NaI | NaCl | NaI | NaCl | NaBr | NaCl | NaI |
| No. of measurements | 120 | 80 | 60 | 70 | 50 | 40 | 40 | 90 | 60 | 10 |
| Mean number of impulses | 22±2·3 | 89±2·1 | 32±1·0 | 57±2·4 | 19±0·5 | 68±1·4 | 27±1·4 | 32±3·0 | 19±4·0 | 70±2·7 |

TABLE 4. EFFECT OF BROMINE, IODINE AND NITRATE IONS ON NUMBER OF NERVE IMPULSES OF STIMULATION OF RETINA WITH ALTERNATION OF LIGHT AND DARK (RHYTHM — 1 SEC)

| | Ringer solutions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | I | | II | | III | | IV | | V |
| | NaCl | NaBr | NaCl | NaI | NaCl | $NaNO_3$ | NaCl | NaBr | $NaNO_3$ |
| No. of measurements | 40 | 70 | 40 | 50 | 70 | 90 | 70 | 50 | 70 |
| Mean number of impulses | 95±1·4 | 109±3·3 | 68±1·9 | 97±3·2 | 60±2·2 | 79±7·7 | 26±1·3 | 30±1·5 | 41±1·3 |

## SUMMARY

(1) Replacement of the Cl ions in Ringer solution bathing the isolated eye of the frog by Br, $NO_3$ and I ions leads to an increase in the number of nerve impulses recorded from the ganglion cells.

(2) In its effect on the activity of the retina, bromine occupies an intermediate position in the lyotropic series of anions: $Cl < Br < NO_3 < I$.


In conclusion, I wish to express my deep appreciation to I. N. Verkhovskaia, Ye. A. Liberman and M. N. Vaintsvaig for valuable advice and assistance in the work.

*Translated by* A. Crozy

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* There is a translation into English of this work.

VOL. 5 1960

# PHYSICAL SIGNIFICANCE OF THE DOSE COEFFICIENT ON THERMAL TREATMENT OF BLOOD*

K. S. Trincher

Institute of Biological Physics, U.S.S.R. Academy of Sciences Moscow

(*Received* 3 *December* 1959)

In his article [1] Pasynskii started from the following equation expressing the functional relation between the quantum yield $\Phi$ on denaturation of the protein molecule by ultra-violet rays and the heat of activation of the denaturation process $\Delta H^*$:

$$\Phi = \Phi_0 \exp(\Delta H^*/E \cdot \log p) \dagger \quad (1)$$

where $E$, is the mean energy of the intramolecular protein bonds, $p$, the probability of rupture of a bond on denaturation, and $\Phi_0$, a proportionality coefficient. On the basis of the experimental findings of other workers for $\Phi$ and $\Delta H^*$, Pasynskii showed that the relation $(\log p)/E$ is approximately constant

* Biofizika **5:** No. 4, 502, 1960.

† This is a mis-quotation of the original Russian reference; $\Phi = \Phi_0 \exp(\Delta H^*/E \cdot \ln p)$.

for all proteins of molecular weight within the limits of $12\times10^3$ (insulin) to $42\times10^6$ (tobacco mosaic virus).

In a previous paper [2] we derived an equation expressing the functional relation between the time of onset of haemolysis in an isotonic alkaline media $t_{haem}(D)$, and the time of preliminary heating of the blood, $(D)$:

$$t_{haem}(D) = t_0\, e^{-k_1 D} \tag{2}$$

where $t_0$, is the time of onset of haemolysis in alkaline medium for blood not previously heated ($D = 0$) and $k_1$, is the dose coefficient. On the basis of our experimental findings for the values $t_0$, $t_{haem}(D)$ and $D$ — the time of heating the blood in mainutes at 50° — we determined a numerical value for the dose coefficient $k_1$.

This value for $k_1 = 0{\cdot}0228 \pm 0{\cdot}0017$; the value $(\log p)/E$, calculated by Pasynskii, is equal to $-0{\cdot}0214$.

Thus, we see that

$$k_1 \simeq -\frac{\log p}{E} \tag{3}$$

The numerical value of the dose coeffient $k_1$ in equation (2) should not depend on the protein concentration or (in our case) on the number of cells subject to heat treatment.

The equality (3) between the dose coefficient $k_1$, from the equation of the kinetics of the haemolytic process after preliminary heating of the blood (2), and the value $-(\log p)/E$ from equation (1) indicates the same physical significance for the dose coefficient $k_1$ and the value $-(\log p)/E$. The value $k_1$ consequently expresses the relation between the mean energy of weak intramolecular bonds in the proteins of the erythrocytes and the probability of their rupture during protein denaturation on heating the blood.

*Translated by* A. CROZY

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* There is a translation into English of this work.

# [BIOPHYSICS IN PRACTICE

# EXPERIENCE WITH THE USE OF MICRODOSES OF GAMMA IRRADIATION IN POULTRY BREEDING*

I. G. KOSTIN

Radiobiological Laboratory of the Tomilin Poultry Factory

(*Received* 25 *March* 1960)

IN the search for stimulatory agents to affect positively the breeding of chicks in incubators and the egg-laying capacity of hens, a large series of experiments has been carried out in the last 3 years using

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microdoses of γ-irradiation. In the period 1957–1959, 90 experimental batches were incubated and more than 75,000 eggs irradiated. The best results were obtained with chronic irradiation of the embryos throughout the entire period of incubation with a total dose of 2·9 r. At this dose the hatching of the chicks rose on average by 2·7 per cent and the egg-laying capacity of the hens grown from the irradiated embryos increased by 7–10 per cent.

As a source of soft γ-rays we used salts of uranium and thorium. For uniformity of irradiation of the eggs within the incubation trays, the uranium and thorium salts were placed in glass tubes mounted in wooden frames built to the same size as the trays. During irradiation the containers with the radioactive substance were placed beneath the tray holding the eggs for incubation.

The following conditions were adhered to in carrying out the experimental work: both the experimental and control batches of eggs were from the same laying-hen and were equalized for weight and number of days after laying. The control groups were kept in two incubators; in an incubator with the experimental batches of and uranium and thorium salts, but at the greatest possible distance from them, and in another identical incubator without a source of radiation.

VOL. 5 1960

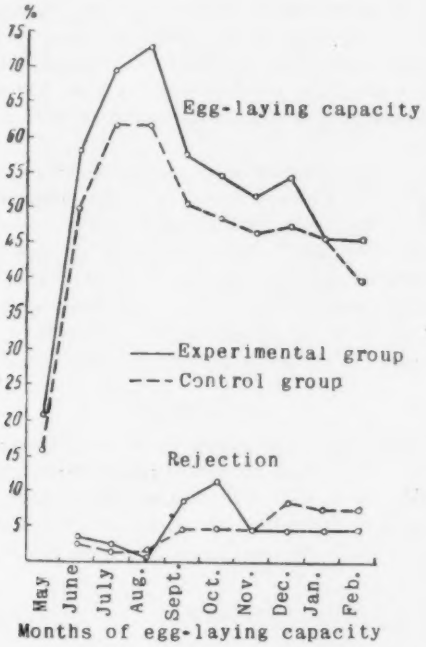


FIG. 1. Curves of egg-laying and rejection of hens of irradiated and control groups (batches No. 14 and 21).

As the observations on the development of the irradiated embryos showed, irradiation gives a positive effect chiefly in the initial stages of development of the embryo (in the first 6 days of incubation) when intensive laying down and vigorous growth of the basic organs of the embryo occurs and in the subsequent completed stage (prior to hatching) when the formation of the incubated chick is finished and it is ready for transition from allantoic oxygen nutrition to atmospheric respiration. When choosing the dose of irradiation, in order to avoid pathological phenomena we decided not to make too great a departure from the natural level of radiation. After prolonged experiments and observations on the results of γ-irradiation we opted for a dose rate of 100 μr/min which has the greatest effect both in

relation to the hatching of the chicks and to the egg-laying capacity of the hens grown from the irradiated embryos. Dose rates of 100 μr/min used continuously during the 20-day incubation ensured that the embryos received a total dose equal to 2·88–2·90 r.

It was found that approximately the same effect could be obtained by irradiating the embryos with the same dose rate only in the first days of incubation, in the period of their greatest radiosensitivity. However, chronic irradiation of the embryos within the limits of this dose gave the most stable positive effect.

Analysing the results obtained in relation to the quality of the incubated egg we conclude that; the higher the biological value of the egg ensuring good hatching of chicks (90 per cent and higher), the less the stimulatory effect derived from irradiation. In this case hatching of the eggs was 2–4 per cent higher on irradiation. When somewhat poorer quality eggs were placed in the incubator (late winter laying, poorer maternal stock, etc.) the difference in the hatching increased by 5–8 per cent in favour of the irradiated batches.

Prolonged observations were made on two batches (No. 14 and 21). In these batches 1208 eggs were placed in the incubator intended for the experiment with chronic irradiation (2·9 r) and the same number of unirradiated eggs served as controls. All the eggs were grouped according to origin, weight and date of laying. Hatching out of healthy chicks in the experimental groups proceeded very satisfactorily giving 2·4 per cent higher hatching figures than for the controls. After withdrawing the 60-day old roosters and forwarding some of the chicks for selection work, 532 chicks were taken for further breeding and subsequent observation on the egg-laying capacities of the experimental and control groups.

The young hens of the experimental groups started to lay eggs on average 4·5 months after hatching whereas it was some 8–10 days later for the controls. In the following months the egg-laying capacity of the experimental groups rose appreciably and exceeded that of the controls by 7–10 per cent. Among the experimental stock of hens individual young ones gave 27–30 eggs per month, but it was noted that in a certain (small) number of such hens the egg-laying fell off after 3–4 months and the agricultural norms made it necessary to reject them as is obvious from the diagram.

The figure shows that microdoses of γ-irradiation may serve as one of the factors raising both the hatching of the chicks and the egg-laying capacity of young hens.

## DISCUSSION

The economic effect of irradiation of eggs during the incubation period may be very considerable. The Tomilin Poultry Factory yearly places 2·5 million eggs in the incubators and hatches a total of more than two million chicks. An increase in the hatching out of chicks merely by this factory itself of only 2 per cent would give an additional 40,000 chicks. The Tomilin Factory has an annual production of 45 million eggs. A rise in the total laying of eggs by 10 per cent would give the country another 4·5 million eggs.

*Translated by* A. CROZY

# DISCUSSION

# THERMODYNAMICS OF IRREVERSIBLE PROCESSES AND THE EXCITATION THEORY*

A. K. PRITS

Herzen State Teaching Institute, Leningrad

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VOL. 5 1960

## POSING OF PROBLEM

MUCH work has recently appeared, including the papers of Pasynskii [1] and Vol'kenshtein [2], indicating the possibility of applying the thermodynamics of irreversible processes to living systems.

Prigogine and Wiame [3] have investigated the application of steady state theory to biological phenomena. This investigation established a relation for the entropy of biological systems.

We shall consider living tissue as an open system able to exchange matter and energy with the environment. Any state, including the state of living tissue, can be characterized by its entropy. In the usual steady state which corresponds to the state of "rest" of the tissue, its entropy is a constant magnitude; positive appearance of entropy

$$\sigma_i = \frac{d_i S}{dt},$$

being the result of a large number of processes developing irreversibly within the system, is compensated for by the negative transmission of entropy from without through exchange with the enviroment. Any living system receives substances of higher organization and gives off to the surrounding space less organized ones. This degradation maintains the steady state of the living system. A condition for a steady process occurring in an open system is constancy of its entropy

$$\frac{dS}{dt} = \frac{d_i S}{dt} + \frac{d_e S}{dt} = 0. \tag{1}$$

Here, the positive appearance of entropy $\sigma_i = d_i S/dt$ may be written in the following manner

$$\sigma_i = \frac{d_i S}{dt} = \frac{1}{T} \sum_{i=1}^{r} A_i I_i. \tag{2}$$

Expression (2) represents the sum of the products of the velocities of chemical reactions $I_i$ and the "forces" of the chemical affinity $A_i$ and $T$ is the absolute temperature.

The negative appearance of entropy $\sigma_e = d_e S/dt$ can be written in the form [6]:

$$\sigma_e = \frac{d'_e S}{dt} + \frac{d''_e S}{dt},$$

where $d'_e S/dt$ is the negative appearance of entropy due to heat exchange with the environment and $d'' S/dt$ is the negative appearance of entropy due to metabolism.

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Stimulating living tissue results in disturbance of the steady state. If the stimulation is sufficiently great, muscle tissue responds to it with change in shape and size, i.e. starts to contract. It is well known that contraction of tissue is caused by the onset of definite chemical processes within the tissue.

If we take this as our starting point, then we may consider that the onset of chemical reactions determining the contraction of muscle tissue, e.g. disintegration of ATP and associated reactions, is determined by the change in the state of the tissue which originates as the result of stimulation by a nerve impulse, electrical current, mechanical treatment, etc, for in the absence of such stimuli the tissue never starts to contract.

Consequently, the results of stimulation is change in the state of the system, i.e. change in its entropy. But change in entropy implies change in the given steady state characterized by definite positive and negative appearances of entropy. Excitation of a living system, which we regard as open, may be looked on as its passage from the given steady state corresponding to its state of "rest" into an excited state determined by other values of the positive appearance of entropy $\sigma_i$ and negative appearance of entropy $\sigma_e$.

Therefore, the excitation of living tissue may be characterized by the difference between the negative appearance of entropy in the excited state and in the state of "rest".

This can be written:

$$\Delta\sigma_e = \sigma_e - \sigma_{e0}. \tag{3}$$

Here, $\sigma_e$ is the negative appearance of entropy in the excited state and $\sigma_{e0}$, in the state at "rest". The value $d\sigma_e/dt$ characterizes the rate of excitation.

We assume that for the muscle tissue to start to contract (threshold effect) its entropy must change by a constant value specific for a given tissue in given conditions.

This corresponds to a definite change in $\sigma_e$ and our postulate may be written in the form:

$$\Delta\sigma_e = \sigma_e - \sigma_{e0} = \text{const.} \tag{4}$$

## RELATION BETWEEN THRESHOLD VOLTAGE OF ELECTRICAL STIMULATION AND ITS DURATION

The characteristics introduced by us for the excitation processes make it possible to consider the relation between the d.c. voltage at the make and the time needed to obtain a threshold effect.

We shall proceed from the electrical model of muscle tissue represented in Fig. 1. agreeing with Rance [4] and Galleotti [5] that the tissue structure determines its resistance and capacitance.

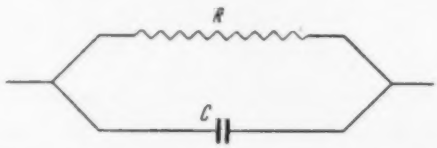


FIG. 1. (R) Ohmic resistance of the muscle tissue; (C) its capacitance.

Change in the appearance of entropy due to switch on of the electrical current is equal [6] to:

$$\Delta\sigma_e = \frac{1}{T} I^2 R, \tag{5}$$

where $I$ is the strength of current passing through the active resistance of the tissue $R$ and $T$, the absolute temperature. After composing and solving Kirchoff's equations, we obtain the following value for the strength of current traversing the active resistance of the muscle:

$$I = I_0 \left[ 1 - e^{-\frac{1}{C}\left(\frac{1}{R} + \frac{1}{R_i}\right) t} \right]. \tag{6}$$

where $R_1$ is the sum of the resistances of the leads and the source of the current, C, the capacitance of the tissue, and $I_0$ equal to:

$$I_0 = \frac{E_0}{R+R_1}, \tag{7}$$

where $E_0$, the e.m.f. of the current source.

Using formula (5) and postulate (4), we may write:

$$\frac{1}{T}\frac{E_0^2 R}{(R+R_1)^2}\left[1 - e^{-\frac{1}{C}\left(\frac{1}{R}+\frac{1}{R_1}\right)t}\right]^2 = Q, \tag{8}$$

where $Q$ is a constant value for the given muscle tissue in given conditions.

Expression (8) may be rewritten thus:

$$E_0\left[1 - e^{-\frac{1}{C}\left(\frac{1}{R}+\frac{1}{R_1}\right)t}\right] = (R+R_1)\sqrt{QR^{-1}T}. \tag{9}$$

VOL. 5 1960

Formula (9) reflects the relation between the voltage and time necessary to obtain a threshold effect on switching on the electrical current as a function of the electrical characteristics of the tissue and the parameters of the instrument.

The minimum voltage which just suffices to cause a threshold effect for an electrical current of unlimited duration is called the rheobase.

It is clear from (9) that the rheobase is equal to:

$$E_p = (R+R_1)\sqrt{QR^{-1}T}. \tag{10}$$

If we assume that $R_1 \gg R$, as is true for nearly all the instruments determining the "strength-duration" relationship, then

$$E_p = R_1\sqrt{QR^{-1}T}. \tag{11}$$

The equality (11) gives the rheobase as a function of temperature. If we make use of the dependence of resistance and capacitance on temperature obtained by Lebedinskii [7] for muscle tissue:

$$R = R_0(1 - \alpha\Delta T), \tag{12}$$

$$C = C_0(1+\beta\Delta T), \tag{13}$$

where $\alpha = 0{\cdot}02$–$0{\cdot}025$ and $\beta = 0{\cdot}03$–$0{\cdot}035$, then the ratio of the rheobases at 17 and 28° will be 0·85. The experiment in [8] gave 0·82.

An ambiguous relation of the threshold voltages on stimulations of different duration was observed long ago [9–11]. This "puzzling ambiguous change in the excitability due to temperature for stimulations of varying duration" [11] is reflected by formula (9). In fact, for low values of $t$,

$$1 - \exp\left\{-\frac{1}{C}\left(\frac{1}{R}+\frac{1}{R_1}\right)t\right\} = \frac{1}{C}\left(\frac{1}{R}+\frac{1}{R_1}\right)t$$

and therefore

$$E_0 t = CR_1 R\sqrt{QR^{-1}T}. \tag{14}$$

apply.

Comparing (9) and (14) we see that the threshold voltages for stimulations of varying duration vary for the same $C$ and $R$ and, consequently, bear a different relationship to the temperature.

It is clear from (11) for $T$ = const. the rheobase $E_p$ = const. and formula (9) may be rewritten:

$$E_0\left[1 - e^{-\frac{1}{C}\left(\frac{1}{R}+\frac{1}{R_1}\right)t}\right] = E_p. \tag{15}$$

## CHRONAXIE AS AN OBJECTIVE CHARACTERISTIC OF THE STATE OF LIVING TISSUE

The question as to whether chronaxie is an objective characteristic of the state of the tissue was the subject of a recent discussion [12, 13]. The discussion centred on the degree to which the chronaxie reflects the state of the tissue and to what extent the construction of the instrument affects the chronaxie.

Formula (15) provides an answer to this question. By chronaxie we mean the time necessary to obtain a threshold effect when the voltage is twice as high as the rheobase. Substituting $E_0 = 2E_p$ in (15), we obtain the value of the chronaxie expressed by the parameters of the instrument and the electrical characteristics of the state of the tissue:

$$\tau = \frac{0{\cdot}7}{\frac{1}{C}\left(\frac{1}{R} + \frac{1}{R_1}\right)}. \tag{16}$$

It is clear from (16) that for the design of the instrument not to affect the value of the chronaxie $R_1 \gg R$ must pertain, i.e. the resistance connected in series with the tissue must be considerably greater than the ohmic resistance of the tissue itself. Then the chronaxie is equal to:

$$\tau = 0{\cdot}7RC. \tag{17}$$

Since $R$ and $C$ are objective characteristics of the state of the tissue, then the chronaxie defined by formula (17) is its objective characteristic. The values for the chronaxie computed from formula (17) agree with the experimental findings.

Taking into account the values for the chronaxie obtained from (16), expression (15) may be rewritten thus:

$$E_0\left(1 - e^{-\frac{0,7}{\tau}t}\right) = E_p. \tag{18}$$

The curve plotted from formula (18) fully fits the experimental curve.

All the formulae obtained also apply to stimulation of tissue by condenser discharges since an exponential pulse can always replace an equivalent square one.

The empirical formula used in physiological studies:

$$i = \frac{a}{t} + b \tag{19}$$

(where $a$ and $b$ are constants) is a particular case of formula (18) for low values of $t$.

It is easy to explain the non-excitability of tissue on passage of a direct current (or with slow change in it) and a current of high frequency. In fact, passage of a direct current is a steady process and $\Delta\sigma_e = 0$. For a current of sufficiently high frequency, the strength of current passing through the active resistance of the tissue is low and not able to produce the contraction effect, i.e. take the system out of the steady state.

## SUMMARY

The present work was an attempt to apply the methods of theoretical physics to the problem of the relation between the voltage of an electrical current and its duration for threshold effects. On the basis of the general concepts of thermodynamics of open systems and the electrical circuit determined by the structure of the tissue, it has been possible to obtain an explanation of the experimental findings relating to threshold effect.

(1) Physical characteristics of the excitation process have been introduced.

(2) An experimentally-confirmed relation has been obtained between the e.m.f. of the current source and the time necessary to obtain the threshold effect as a function of the parameters of the instrument, the electrical characteristics of the tissue and the absolute temperature.

(3) The dependence of the rheobase on temperature has been obtained.

(4) The expression of chronaxie as a function of the electrical characteristics of the tissue and the parameters of the instrument is given.

(5) The "insensitivity" of muscle tissue to direct and alternating current of high frequency is explained.

The author expresses his thanks to Prof. M. V. Vol'kenshtein for guidance in the work.

*Translated by* A. CROZY

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VOL. 5 1960

# CURRENT EVENTS

## CONFERENCE ON THE BIOPHYSICS OF ERYTHROCYTES*

INTEREST in the study of erythrocytes is very great, this is due to the fact that the erythrocyte, as a cell with relatively simple organization, is a convenient object in the methodological sense and is of theoretical and practical importance in investigations of a number of general biological problems. Study of the erythrocytes is also important because the state of the red cell plays an essential role in clinical medicine. The problem of blood preservation, of great practical interest, also requires thorough study of the elementary biochemical and physicochemical processes in erythrocytes.

However, to date in our country no special conferences or meetings devoted to the study of erythrocytes had been held. Yet the need for such a gathering was obvious. Therefore, we must applaud the initiative of the Biophysics Laboratory of the Institute of Physics, U.S.S.R. Academy of Sciences in convening a conference on the biophysics of erythrocytes.

The Conference was held in Krasnoyarsk from 3 to 9 July 1959. It was attended by biophysicists, biochemists, physicists, physiologists and clinicians from 18 scientific institutes from Krasnoyarsk, Moscow, Leningrad, Sverdlovsk, Irkutsk, Tomsk and other towns. The Conference discussed 56 reports on the biochemistry, physico-chemical properties, structure and functions of erythrocytes in normal and pathological conditions as well as on the comparative physiology and morphology of erythrocytes and the effect of radiation on these cells. Further, a number of papers dealt with the

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importance of the method of erythrograms, investigation of blood preservation and other problems in the study of blood. Mere enumeration of the problems discussed shows that the Conference went beyond the bounds of the biophysics of erythrocytes. In this respect, the variety of problems examined at the Conference seems to us to be a positive step, since it gave rise to a keen and useful exchange of experience and opinions between investigators engaged in erythrocyte research, but working on different problems and using different methods.

A group of reports was devoted to the biophysical study of erythrocytes by means of the erythrogram method — a differential method of analysis of red blood devized in the Biophysics Laboratory of the Institute of Physics, U.S.S.R. Academy of Sciences. During the Conference repeated reference was made to the high practical value of this method. While a differential characterization of white blood is common clinical practice, normal erythrocytes (normocytes) were, until recently, taken as homogenous. The erythrogram method makes it possible to distinguish normocytes by their resistance to haemolysis. The physicochemical and physiological bases of the erythrogram method were outlined in the papers presented by the originators of this method — I. A. Terskov and I. I. Gitel'zon. They gave new material to the Conference showing that the osmotic resistance of erythrocytes is determined by the age. In the view of clinicians, these findings may be of importance in medical practice.

Members of the Biophysics Laboratory of the Institute of Physics, U.S.S.R. Academy of Sciences delivered a series of papers on further improvements of the erythrogram method. In particular, the paper by V. T. Poyetova gave information on the development of this method as applied to the study of immune haemolysis.

B. N. Tarusov and associates (Biophysics Department of the Moscow State University) reported on the application of the erythrogram method to study the activity of tissue haemolysins after irradiation of animals. The clinical application of the erythrogram method was reflected in the report of A. I. Vorob'ev (Central Post Graduate Medical Institute, Moscow).

P. S. Vasil'ev and M. P. Petrova (Central Institute of Haematology and Blood Transfusion, Moscow) have investigated changes in the physicochemical properties of erythrocytes under the influence of haemolytic factors and also during blood preservation. They showed that during prolonged storage of blood, destruction of the erythrocyte structure passes roughly through the same stages as on haemolysis due to haemolytic factors. They have distinguished a stage, which they term stromoporosis, which precedes stromatolysis and is characterized by a high electrical conductivity with retention of the morphological integrity of the cell.

The results of investigations of the physicochemical properties of the blood and erythrocytes were also discussed in the reports of Iu. A. Moskalenko (Sechenov Institute of Evolutionary Physiology, Leningrad) and G. A. Dvorkin (Institute of Biophysics, Academy of Sciences of the U.S.S.R., Moscow). The former showed that the electrical parameters of the blood — electrical conductivity and dielectrical losses — are largely determined by the properties of the erythrocytes and the velocity of blood flow. The author has established a relation between the electrical properties of the blood, the size of the charge on the erythrocytes and their number per unit volume.

G. A. Dvorkin presented information gained from study of surface adenosine polyphosphatase and the electrokinetic potential of erythrocytes. It was shown that ultrasonics may cause a considerable increase in the activity of this enzyme. Analysis of such findings observed in investigations of other surface enzymes has led to the assumption that some enzymes are in the bound inactive state in the cell. It has also been demonstrated that at low ultrasonic intensities there may occur in the cell subtle physicochemical changes not accompanied by gross mechanical destruction. The report stressed that ultrasonics may be utilized in investigation of a number of biological problems.

The Conference also heard reports on study of haemoglobin and its derivatives. In the papers of V. G. Kovrov and M. S. Levinson (Institute of Physics, U.S.S.R. Academy of Sciences) they pointed to the heterogeneity of haemoglobin in erythrocytes and its changes with age. In the discussion on these reports reference was made to the need for further experiments with crystalline haemoglobin and also to the desirability of investigating haemoglobin in certain blood diseases. The authors also reported on the effect of ultrasonics on aqueous haemoglobin solutions.

VOL
5
196

V. V. Ovchinnikov (Irkutsk Medical Institute) reported modern ideas on the mechanism of biological reduction of methaemoglobin. In the discussion, mention was made of the importance of defining the conditions determining oxidation and oxygenation of haemoglobin.

P. A. Shershnev (Irkutsk Medical Institute) gave a review of certain unsolved problems in the biochemistry of anuclear erythrocytes. He emphasized the importance of study of the pentosephosphate cycle of oxidation of glucose in erythrocytes. It was also noted that there is as yet no clear appreciation of the mechanism of "selection" of anuclear erythrocytes entering the general circulation in normal conditions. At the same time, the earlier nuclear forms usually do not penetrate into the blood stream. Such selection, in the view of the speaker, cannot be explained solely by the difference in the size of mature and immature erythrocyte forms. There must also be biochemical differences between them. An interesting point made by the speaker was that enzymes within the erythrocyte may be bound in structural formations with haemoglobin as a constituent. Some ideas were also advanced on the link between haemoglobin and the process of glycolysis in red blood cells. It has been shown in the author's laboratory that under certain conditions the intensity of glycolysis in erythrocytes containing carboxyhaemoglobin changes as compared with controls.

VOL. 5 1960

The report of A. I. Fedorova (Irkutsk Medical Institute) dealt with permeability of anuclear erythrocytes to di-sodium phosphate, methionine and cysteine labelled with radioisotopes. The permeability for these substances diminishes on inhibition of glycolysis by various poisons. Speakers in the discussion pointed to the difficulty of interpreting the changes observed.

G. A. Myskkina (Irkutsk Medical Institute) reported on the binding of yeast invertase by erythrocytes. A correlation was established between glycolysis and this process. The speaker noted that binding of invertase and amino acids by erythrocytes does not constitute genuine physicochemical adsorption and is a process involving many metabolic reactions. Extensive experimental material on the enzymes of erythrocytes in normal conditions and in cancer was presented by L. D. Koshevnik and associates (Tomsk Medical Institute).

Some papers discussed the problems of radiation damage to erythrocytes.

Iu. A. Kriger and I. M. Parkhomenko (Moscow State University) pointed out that erythrocytes are sensitive to relatively low sublytic doses of γ-rays (10,000 r). After irradiation the sensitivity of the erythrocytes, in particular in relation to photodynamic action, is changed. The speakers saw this as the result of summation of the effect of free radicals formed by radiation and by photodynamic action. Irradiation has also been shown to produce a considerable fall in the percentage reversion after hypotonic haemolysis of irradiated erythrocytes. In the view of the authors, this indicates weakening of the bonds between the components of the erythrocyte stroma in response to irradiation. This assumption has been confirmed in earlier works of the speakers.

The report of Ye. D. Gol'dberg (Tomsk Medical Institute) analysed the distinctive damage to erythrocytes on whole body irradiation of animals in a betatron. As well as changes in the erythrocytes, crystals of haemoglobin have been detected in the blood and bone marrow.

The effect of ionizing radiation on erythrocytes was also discussed in the paper of P. S. Vasil'ev and M. P. Petrova (Central Institute of Haematology and Blood Transfusion, Moscow). V. P. Makarov (Institute of Physics, U.S.S.R. Academy of Sciences) reported on the development of irradiation aftereffect in erythrocytes.

The results of investigations of normal and pathological structures of anuclear erythrocytes were presented in the report of D. I. Gol'dberg (Tomsk Medical Institute). Application of the method of haemolytic staining of blood specimens has established histologically the presence of a membrane in erythrocytes. The dimensions and morphological changes in erythrocytes in anaemia of varied origin had also been studied.

L. I. Irzhak and L. I. Radzinskaia reported on the ontogenetic and phylogenetic changes in red blood cells. Discussion on these reports brought out an interesting parallelism between the morphological findings of the authors and the results of the biophysical investigation of erythrocytes reported by I. I. Gitel'zon (Institute of Physics, U.S.S.R. Academy of Sciences).

Several papers read to the Conference were concerned with the methods of investigating blood — e.s.r. determination, optical methods of investigating blood serum (S. G. Bogomolov) the cuprosulphate method of blood examination (A. S. Ivanovskii) etc. The report of G. R. Baluyeva, and L. S. Solov'ev (Institute of Physics, U.S.S.R. Academy of Sciences) contained preliminary data on the infra-red spectra of plasma and whole blood. The discussion brought out the difficulty of interpreting the findings presented. In addition, the desirability of study of serum proteins was underlined.

The Conference took stock of the work done on the biophysics of erythrocytes, helped to clarify the most pressing problems and appraised a number of new methods for study of red blood. The most urgent problems were recognized as: investigation of the structure, physico-chemistry and biochemistry of anuclear erythrocytes in normal and pathological conditions, investigation of types and fractions of haemoglobin, study of the qualitative differences in normal erythrocytes and the mechanism of their death, immuno-haematological work and also investigation of the response of erythrocytes to the action of ionizing radiation.

The Conference felt it necessary to set up a specialist haematological centre in Krasnoyarsk. It was also deemed desirable to form a permanent commission on biophysics within the framework of the U.S.S.R. Academy of Sciences.

The proceedings of the Conference will be published. There was unanimous agreement that the Conference had been extremely well organized and delegates expressed their appreciation of the warm welcome accorded them.

G. A. Dvorkin
*Translated by* A. Crozy

VOL
5
196

## REVIEW

R. Stacy, D. Williams, R. Worden and R. McMorris, Essentials of Biological and Medical Physics. Translated from the English under the editorship of Prof. L. A. Tumerman (Foreign Literature Publishing House, Moscow, 1959)

As the authors point out in the preface to the original, the book was written on the basis of 4 years of teaching biophysics to the students of the higher courses of the biological and physical faculties of the University of Ohio (U.S.A.). The book is a text-book for students, however, since it systematizes extensive material drawn from many original sources, the authors also consider it useful for teachers of this discipline and, in general, for physicists, biologists and medical men interested in the problems of biophysics.

As the book has the title "Essentials of Biological Physics", it is interesting to see how the authors define the subject matter and limits of this comparatively new science. In the preface to their work the authors point out that, as they see it, biophysics embraces the following three groups of problems (1) use of physics to explain biological phenomena; (2) laws of the action of the physical environmental medium on the organism; and (3) use of physical techniques and instruments for the investigation of biological systems.

It is possible that such a definition of biophysics was acceptable in the period in which the authors were preparing their manuscript for the press (the original was published in 1955). Since then 5 years have passed during which there has been very substantial development of biophysics and such a definition cannot satisfy us today.

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From the contemporary point of view, biophysics does not simply "explain" biological phenomena from the physical standpoint, but constitutes the science of the physicochemical bases of vital phenomena and as such, has its own body of problems. The problems of biophysics were indicated in his time by P. P. Lazarev and are, for example, problems of growth and development of organisms, thermodynamics and kinetics of vital phenomena, interconversion of various forms of energy in the body, phenomena of excitation in tissues and organs, and so on. These problems have certainly not lost their importance but rather has their range been considerably extended. At the same time, because modern physics makes possible penetration not only of the living cell but the essence of the molecular processes within it, the character of the approach to the solution of these problems has gained considerably in depth.

True, modern biophysics also encompasses problems of the interaction of the body and its individual systems and organs with the physical environment. However, these problems being the biophysical introduction to different branches of physiology, patho-physiology and certain specialist medical disciplines are better defined as elementary, as it were "minor" biophysics, whereas the essential problems mentioned above form, figuratively speaking, "major" biophysics.

A third applied branch of biophysics can be considered to be the extensive field of biophysical methods of investigating various functions of the body.

We shall see how fat the material outlined in the book under review answers to these modern ideas of biophysics.

Part I is entitled "Fundamental concepts of biophysics" and begins with a Chapter headed "Biophysical systems". The narrative contains a definition of the subject matter of biophysics which is, however, confined to the observation that this science is concerned with fringe problems of physics, biology and medicine. Then, as from the second page, the authors turn to an outline of methodological problems, for example, the elements of a research experiment, the limits of application of theory and the use of theoretical models, the mathematical analysis of biophysical systems, etc. Only in the final Chapter of this Part do the authors, in reverting to the problem of the components of biophysical systems, analyse the physicochemical phenomena in tissue cells especially in the boundary structures between them.

On acquainting himself with this Part, the reader feels legitimate dissatisfaction as the essential problems of biophysical theory receive nothing like adequate treatment. Nor do we agree with the authors that biophysics is concerned with the boundary region between physics, biology and *medicine* (my emphasis — N. L.). Biophysical problems are of undoubted interest in medicine, but they are general biological problems in their own right. The description bordering with medicine can only apply to the elementary problems of biophysics principally concerned with the laws of interaction of the human body and external environment.

Part II — "Mechanical biophysical systems" covers a quite wide range of problems which can be placed into two groups: (1) theoretical problems connected with the nature of the mechanical properties of cell protoplasm and particularly with the mechanical properties of muscle fibre; and (2) the technological problems of biomechanics, e.g. strength of bone tissue and its experimental determination, certain elements of the mechanics of the supportive-motor apparatus, action of forces of inertia on the body and, finally, pathology and therapy of the bone-muscle system.

The mechanism of muscular contraction is based on a consideration of the information on the mechanics of elastomers as exemplified by the properties of rubber. Applying the findings of statistical mechanics the authors compute the fundamental characteristic of rubber — the relation between elongation and tension. They then experimentally establish the possibility of applying the results obtained to a model protein (myosin) filament and also to the isolated muscle fibre.

There follows an analysis of the visco-elastic properties of muscle fibre and finally, an explanation of the mechanism of muscle fibre contraction through change (folding or coiling into a spiral) in the molecular structure of the fibre.

The authors give a fairly full outline of the mechanical properties of soft tissue and the mechanism of muscular contraction. Drawing on comparative data from the field of polymer mechanics they not

only reach definite conclusions in this particular case, but indicate the principle of approach to the solution of such problems. A weakness in the authors' theory of muscular contraction mechanics is that almost no reference is made to the energy aspect of the phenomenon — mechanism of conversion of the internal energy of the chemical compounds involved in the process into the mechanical work performed by the muscle. Moreover, literally in one sentence, mention is made of such an extremely interesting problem as the production of a mechanical mover [?] using (by analogy with the work of the muscle) contraction of an elastomer under the action of special chemical substances — activators (plasticizers).

In considering the mechanical properties of soft tissues, a factor of great interest is their histological structure in relation to the acting load, i.e. the problem of histobiomechanics, but this problem is dealt with only in relation to bony tissue, much attention being focused on testing of its mechanical properties.

The description of the mechanical properties of the supportive-motor apparatus is confined in the book merely to elementary statics (lever equilibrium). The problems of the mechanics, articulations, kinematics and dynamics of movement are not discussed.

The problem of the pathology and therapy of the bone-muscle system is included in the section on biomechanics. It would seem that such a section ought to contain a biophysical analysis of the pathogenesis of particular lesions of the system and the corresponding recommendations for their pathogenetic therapy. In fact, the narrative is confined to a summary of diseases and procedures for their treatment. For the biophysicist this information is abtruse and for the clinician too cursory and elementary, consequently this chapter appears as an unwanted inclusion.

VOL
5
196

The last Chapter in this Part deals with the effect of the action of inertial forces on living tissues. This problem, belonging in principle to biophysics, is usually outlined in an applied form in aviation medicine. In this case, most of the exposition is devoted to a description of the apparatus and methods of testing human endurance of such effects rather than to theoretical problems.

Part III is given over to the heat and thermodynamics of vital processes. First the authors give brief definitions of thermodynamic potentials (entropy, Helmholz free energy, Gibbs function, etc.). These concepts are thereupon dropped and the approach to the living body as a thermodynamic system is regarded solely from the view point of the relation between the rate of chemical reactions in it and the temperature and participation of catalysts. As regards the problems of this subject no more is said and the subsequent narrative is devoted to the known information on physical mechanisms of heat loss and temperature regulation.

The last chapters of this part are devoted to human endurance of high and low temperatures and the application of heat in therapeutics.

Part IV bears the heading "Bioacoustics" and contains three sets of problems: (a) physical theory of acoustic systems; (b) acoustical properties of body tissues; and (c) the biophysics of perception of sound. As a whole, the material covered corresponds to the problems of "elementary" biophysics. The only point to be made is that too little is said on such concepts as loudness and timbre of sound and also on the harmonic spectrum.

Very little is said on the primary effect of ultra-sonics on the body tissues while at the same time undue attention is paid to the purely physical problems of vibratory motion.

Part V is devoted to the biophysics of light although for some reason or another it is headed "Biophysical studies on the effect of light on organisms" [*sic*]. It firstly outlines the nature of light, the laws of its absorption by matter and the primary effect — activation of molecules. As particular examples of the reactions resulting from this activation a description is given of photodynamic action, photosynthesis and the general biological response to light of various organisms. A subsequent Chapter contains basic information from the biophysics of visual processes and is concerned with the mechanism of visual sensation, sensitivity of the eye and the theory of colour vision.

The biophysical problems, in particular, the optical system of the eye, birefringence of biological objects and bioluminescence are mixed up with purely physical problems which the authors apparently assign to the field of medical physics. These problems begin with a description of the elementary concepts of geometric optics and go on to a discussion of the physical basis of microscopy, its special forms

(ultra-violet and electron), the optical activity of matter and, finally, X-ray diffraction. Of the 60 pages given over to these problems no less than 40 are repetition of the contents of a physics text-book.

Part VI is entitled "Gas physics in biology" and gives a fairly detailed analysis of the problems of the mechanics of pulmonary respiration and especially gaseous exchange in the lungs. The problems of exchange of gases in high altitude flight and caisson disease [*sic*] are dealt with more briefly.

Material is contained in this Part which migh t be described as an elementary biophysical introduction to pulmonary respiration in physiology.

Part VII is headed "Fluid systems in biology" and is concerned with the biophysical problems of blood circulation with information on the movement of viscous fluids along tubes and an analysis of the work of the heart and the general physical laws of movement of the blood in the vascular system.

In our view, these problems by no means exchaust the significance of (repeating the heading) fluid systems in biology.

Fluid systems, in the wide sense, are the basis of all biophysics which is even sometimes called physicochemical biology. The authors themselves in the introductory Part defined "components of the biophysical systems" in the form of the fluid colloidal system of the cell referring also to the physicochemical phenomena (surface tension, osmosis and diffusion) underlying cell metabolism. However, these problems are not developed in the subsequent pages. In this respect the authors do not fulfil their promises made in the introductory Part. For example, on p. 33 (Russian text) we read: "Of fundamental importance in the understanding of biological systems is a knowledge of the processes by which chemical materials are distributed within a cell or tissue. It is often true that the rate of a reaction governing the behaviour of a system depends not on the system itself, but rather on the speed with which needed materials are transferred into the active cells and the speed with which activity products are removed. *From time to time in the remainder of this book such situations will arise and will be pointed out*". (My italics — N. L.).

OL.
5
960

However, apart from the main law of diffusion presented in general terms and the theoretical explanation of the nature of osmosis we find nothing of this theme in the book, this is clearly not good enough.

Part VIII has the title "Electrical phenomena in biology" [*sic*] and under this very promising title the following particular problems are reviewed: electrical properties of tissues and cells, mechanism of origin of biopotentials and methods of recording them, cell response to tissue excitation, effects of an electrical current on animals and, finally, the principles of electrodiagnosis, electrotherapy and diathermy.

The electrical properties of body tissues are considered in a rather simplified form — from the viewpoint of the properties of electrolytic solutions separated by semi-permeable membranes. A considerable place is given over to a consideration of the impedance of tissue elements and its measurement.

The physical mechanism of formation of the resting biopotentials is outlined on the basis of the Donnan equation determining the equilibrium of ionic concentrations on either side of the semipermeable membrane.

The cell response to stimulation and the origin of the action biopotentials is considered from the standpoint of the excitation theory developed by Hill. The work in this field of Nernst, Leb and Lazarev is not mentioned.

In the Chapter "Recording of potentials from the surface of the animal body", an interesting, though extremely brief, explanation is given of the mechanism of production of the spikes of electrocardiograms and the principle of electrocardiography. For reasons not easily understood this Chapter contains a paragraph on the electric organs of fishes.

In the Chapter dealing with the effects of electric current on the body and its use for therapeutic purposes, instead of disclosing the mechanism of action of an electrical current on cells and tissues (within the competence of biophysics, even according to the authors own definition) the text gives rather rambling arguments on the relation between voltage, resistance and strength of current and electrical injury, electric shocks, cardiac fibrillation and high-frequency heating. All this is common knowledge with no direct bearing on biophysics.

"Applications of Nuclear Physics in Biology" is the title of Part IX. It should be mentioned that there exists a tradition according to which purely physical problems of the structure of the nucleus and the nature of radioactive emissions, etc come under biophysics and are sometimes substituted for the basic, truly biophysical problems of the mechanism of the primary action of ionizing radiations on living tissue. The authors of the book under review follow this tradition. The first 36 pages (Russian text) outline the purely physical problems of nuclear physics. The following 22 pages (Russian text) are devoted, admittedly, to the biological effects of ionizing radiations but the symptomatology of radiation injuries in various cases is given fuller attention. The problems described in this Part of the book are obviously dated.

"Theoretical Biophysics" which constitutes Part X concludes the book and is of the nature of a short epilogue in which the authors only recall the reader's attention to the possiblility of application in biophysics of the method of modelling pathological phenomena by physical agents and, in particular, construction of cybernetic systems reproducing given body functions.

In concluding this short review of the contents of this book, it may be stated that an unquestionable merit of the book is its considerable store of factual material on various branches of biophysics. A fundamental defect of the monograph is that it does not present biophysics as a science from the modern point of view. It does not force the reader to ponder on any of the problems of "major" biophysics". In it the reader will come across much useful material on various problems but all refer to the field of elementary "minor" biophysics. To a certain extent only the question of the mechanical properties of muscle fibres is of a "problem" character.

Fault can also be found with the construction of the book itself which corresponds to the subdivisions of a physics course (mechanics, acoustics, electricity, light, etc.) and this contributes to the breaking down of the problems of biophysics into smaller elementary problems relating to one or other of these subdivisions.

It should be noted that this book was translated 5 years after its publication. It is possible that at that time we should have made more modest demands on it.

Exception can also be taken to the extremely analytical approach of the authors to all the problems considered. In all the sections consideration is given only to the body elements (cells, cell systems, tissue) with scanty attention to synthesis of the results obtained for the whole body. If phenomena in the whole body are considered, then the authors pass from the field of biophysics to that of physiology.

It must also be noted that the authors treat all the problems raised with reference to the human body and in stressing the medical aspects introduce subdivisions on the treatment of human diseases. This limits the exposition and many general biological problems are touched upon cursorily in passing.

Considerable space is occupied by purely physical problems. Apparently the authors assign them to the field of medical physics. We shall not enter into a definition of this concept but merely say that the elements of physics presented in the book do not make it any the more profound and distinctly impair the scope of the exposition of essential biophysical problems.

The considerable unevenness of the mathematical interpretation of physical phenomena should be noted. Some receive elementary treatment, while for others higher mathematics such as vector analysis, field theory, etc are adopted which may well go beyond the confines of the reader's mathematical training.

Reference is made mainly to American literature. This defect has been put right by the editor o the translation who appends notes giving the appropriate Russian literature on all the main problems

In conclusion, we would note that for the category of readers to whom the authors recommend the book it will be very useful. However, in utilizing the book it must be borne in mind that the material contained in it does not exhaust but even does not fully characterize present-day biophysics and must therefore be considered rather as the "elements" of biophysics than its "essentials".

Production of an exhaustive work on the essentials of biophysics is a task still to be undertaken.

N. M. LIVENTSEV
*Translated by* A. CROZY

# CHANGE IN THE SURFACE PROPERTIES OF IRRADIATED DEOXYRIBONUCLEOPROTEIN AND DEOXYRIBONUCLEIC ACID*

A. M. TONGUR and A. G. PASYNSKII

Bakh Institute of Biochemistry, Moscow

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VOL. 5 1960

NUCLEIC acids and nucleoproteins together with the lipoproteins play an important rôle in various intracellular structures and in the formation of molecular interfaces in the protoplasm. Irradiation results in destruction of these compounds, expressed most sharply for the nucleic acids. The result is chemical and structural changes in the properties of the nucleic acids and nucleoproteins in the cell and in the various internal interfaces of the cell. It should be noted that the surface properties of the nucleic acids and nucleoproteins have still received nothing like sufficient attention. In particular, the conditions of formation of DNA monolayers have been investigated in only two studies; Miura, Imahori and Watanabe [1] studied apurine DNA, the purity of the sample of DNA was not stated in the paper by James and Mazia [2]; the effect of irradiation was investigated in neither case.

It was therefore of interest to make a study in the present work of the ability of DNA and DNP to form monolayers and the influence of X-irradiation of these substances on their properties in monolayers.

## EXPERIMENTAL

*(1) Experiments with deoxyribonucleoprotein.* The nucleoprotein was isolated from the calf thyroid gland by the technique of Mirsky and Polister.

The two samples of nucleoprotein studied had the following characteristics: ratio of nitrogen to phosphorus (N/P) 3·8 and 3·7; molecular weights as determined by the method of viscometry [3] 4·5 and $8 \times 10^6$ respectively.

The monolayer of nucleoprotein was obtained in a Langmuir apparatus by the application of drops, from a specially calibrated microsyringe, of 0·04 % nucleoprotein in 1 M NaCl in 38% aqueous solution of ammonium sulphate. This backing was chosen in order to reduce the solubility of the nucleoprotein applied and to facilitate its spread.

The thickness of the monolayer was determined at a lateral pressure of 0·1 dyne/cm by two means. In the first method, the size of the surface occupied at this pressure

* Biofizika **5**: No. 5, 517–522, 1960.

by a known amount of the nuceloprotein applied was determined; the thickness of the monolayer obtained in this way was $20 \pm 1$ Å. With the second method, part of the monolayer was removed on a silver mesh with known surface area (270 $cm^2$), the area of the monolayer removed on the mesh corresponded to the difference in the areas of the monolayer (with the same compression) before and after removal. Thus, the area of the monolayer removed was equal to the area of the mesh. Dissolved in 0·5 l. of 1 M NaCl, the solution was evaporated to dryness in a porcelain dish over a water bath, the dried residue and washings dialysed against 0·5 l. of distilled water and the concentration of nucleoprotein determined in the dialysate by a spectrophotometric method [4]: this gave the thickness of the monolayer as $28 \pm 1$ Å which is probably somewhat in excess of the real figure.

Nevertheless, from the results obtained by both methods it follows that in these conditions the nucleoprotein actually formed a monomolecular layer and that the molecules of the nucleoprotein (including both components — nucleic acid and protein) lie in a horizontal plane at the interface, otherwise the thickness of the layer would have been considerably greater.

In experiments with X-irradiation, the solutions of nucleoproteins (0·04% in 1 M NaCl) were irradiated before application to the surface by a near-focus BF tube (150 kV; 8 mA; $\lambda \sim 0{\cdot}6$ Å) with a dose rate of 1130–16,900 r/min. The solution for irradiation was placed in a polymethacrylate dish and cooled with water during irradiation. The height of the liquid layer was 0·8 cm.

As a result of irradiation with a dose of 300–600,000 r, the viscosity of the nucleoprotein solutions was greatly decreased and the molecular weight, measured by the viscosimetric method, diminished in both samples down to about 20,000.

Curves were obtained for both the unirradiated and irradiated samples of the nucleoprotein relating the area of the monolayers to the pressure for 0·04% solutions in 1 M NaCl (on a Langmuir instrument). The results are given in Fig. 1 which shows that up to a dose of 50,000 r the curves were virtually unchanged as compared with those for the unirradiated nucleoprotein, and only in the $10^5$–$10^6$ r interval were they shifted somewhat to the left, the shape of the curves remaining constant in all cases. A particularly characteristic feature was the parallelism of the curves in the region of condensed films, irrespective of whether they were obtained from the unirradiated nucleoprotein or the heavily destroyed samples. Figure 1 shows that for a pressure of 20 dyne/cm, the unirradiated sample occupied 0·15 $m^2$/mg and that irradiated with a dose of 300,000 r, 0·13 $m^2$/mg; at 10 dyne/cm the figures were respectively 0·19 and 0·165 $m^2$/mg. Thus, despite the considerable degree of destruction (fall in molecular weight by some tens of times, see Table 1), the area occupied by the nucleoprotein in the monolayer per mg weight remained virtually unchanged. At zero pressure (point of intersection with axis of the abscissa), the area of the unirradiated nucleoprotein (Fig. 1, curve 1) was 0·36 $m^2$/mg and of the irradiated one (curves 4 and 5) 0·30 $m^2$/mg.

In both cases, as can be readily calculated, the thickness of the layer was:

$$d = \frac{10^{-3} \times 0{\cdot}7}{0{\cdot}36 \times 10^4} = 2 \times 10^{-7}\ \text{cm} = 20\text{Å},$$

which, in effect, corresponds to the thickness of the monolayer. As can be seen, this thickness underwent no change on destruction of the nucleoprotein by irradiation.

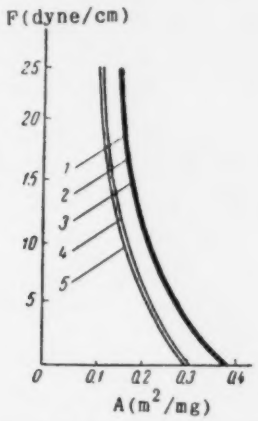


Fig. 1. Curves relating area (A, $m^2$/mg) of monolayers to pressure (F, dyne/cm). (1) Unirradiated deoxynuceloprotein (mol. wt. = $4{\cdot}2 \times 10^6$); (2, 3, 4, and 5, Deoxyribonucleoprotein after irradiation at a dose of $10^4$ r, $5 \times 10^6$ r, $3 \times 10^5$, and $6 \times 10^5$ r respectively.

At the same time, after destruction, the area occupied by one molecule naturally changed (see Table 1).

TABLE 1. MOLECULAR CHANGES IN NUCLEOPROTEINS ON IRRADIATION

| Dose r ($\times 10^3$) | Molecular weight, measured viscometrically | Area of monolayer as % of original sample, under pressure (dyne/cm) | | | Thickness of monolayer (Å) | Area occupied by 1 molecule ($Å^2$) |
|---|---|---|---|---|---|---|
| | | 0 | 10 | 20 | | |
| Deoxyribonucleoprotein (mol. wt. = $4{\cdot}5 \times 10^6$) | | | | | | |
| 0 | $4{\cdot}5 \times 10^6$ | 100 | 100 | 100 | 20 | $2{\cdot}6 \times 10^5$ |
| 10 | $2 \times 10^6$ | 100 | 100 | 100 | 20 | $1{\cdot}2 \times 10^5$ |
| 50 | $3 \times 10^5$ | 100 | 100 | 100 | 20 | $1{\cdot}8 \times 10^5$ |
| 300 | $2 \times 10^4$ | 94 | 91 | 87 | 20 | $2{\cdot}5 \times 10^3$ |
| 600 | $2 \times 10^4$ | 77 | 82 | 89 | 20 | — |
| Deoxyribonucleoprotein (mol. wt. = $8 \times 10^6$) | | | | | | |
| 0 | $8 \times 10^6$ | 100 | 100 | 100 | 20 | $5{\cdot}3 \times 10^5$ |
| 210 | $2 \times 10^5$ | — | — | — | 20 | — |
| 420 | $2 \times 10^4$ | — | — | — | 20 | $2{\cdot}5 \times 10^3$ |
| 900 | $2 \times 10^4$ | 75 | 72 | 79 | 20 | — |

The shift in the curve to the left for high doses of radiation was probably due to some of the more degraded molecules dissolving in the backing solution: change in the composition of this medium appreciably changed the properties of the monolayer. Thus, with a backing consisting of 38% ammonium sulphate and 2% formaldehyde, the area of the nucleoprotein, unirradiated, did not remain constant, as it did for a backing of 38% ammonium sulphate without formaldehyde (Fig. 2, curves 1 and 2),

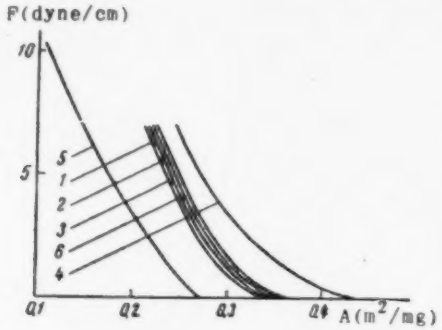


FIG. 2. Curves relating area (A, $m^2$/mg) of monolayers to pressure (F, dyne/cm) of deoxynucleoprotein. (1, 2) Unirradiated deoxyribonucleoprotein with a backing of 38% ammonium sulphate (curve 1 plotted immediately after application of monolayer, curve 2, 1 hr after application); (3, 4) unirradiated deoxyribonucleoprotein with backing of 38% ammonium sulphate and 2% formaldehyde (curve 3 plotted immediately after application of monolayer, curve 4 after 1 hr without pressure); (5, 6) similar curves plotted in same conditions for preparation irradiated with a dose of $10^6$ r.

but was somewhat increased on standing (Fig. 2, curves 3 and 4). It would seem that this process is to be explained by the slow reaction between the formaldehyde and nucleoprotein distorting the native structure of the latter. A similar process was observed for the irradiated nucleoprotein (Fig. 2, curves 5 and 6). Apparently, the interaction with formaldehyde led to an increase in the molecular weight of the nucleoprotein and a reduction in the number of "free" polar groups which improved the conditions for spreading and was accompanied by a certain shift in the curves to the right.

It is interesting to note that all the monolayers of the nucleoprotein spread on a backing of 38% ammonium sulphate, or a mixture of the latter with 2% formaldehyde, showed complete reversibility of the deformation — verified in the curves relating area to pressure which were repeated 10 times in the pressure range from 0 to 10 dyne/cm.

*(2) Experiments with deoxyribonucleic acid.* Deoxyribonucleic acid, isolated from the thymus by the method of Mirsky and Polister, with a molecular weight (measured by the viscosimetric method) of about 2 million and a nitrogen to phosphorus (N/P) ratio of 1·78, did not form a monolayer on application by the drop method to a backing consisting of 1 M NaCl at pH 7 or 38% solution of ammonium sulphate.

The reason for this is the considerable solubility of DNA in these solutions. Therefore, it may be assumed that the findings of James and Mazia [2] on obtaining

a monolayer of DNA in 2 M NaCl are probably to be explained by the inadequate purity of the preparation they used, and the presence in it of admixtures of nucleoprotein (as already stated, the purity of the DNA was not specified in the work of these authors).

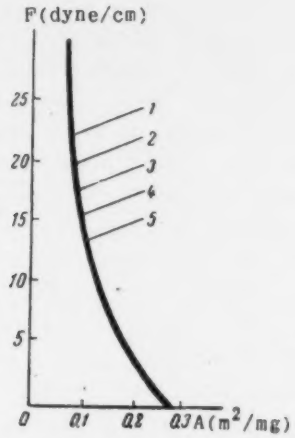


FIG. 3. Curves relating area (A, m²/mg) of monolayers to pressure (F, dyne/cm) for deoxyribonucleic acid. (1) Unirradiated DNA; (2) DNA irradiated with dose of $10^4$ r; (3) DNA irradiated with dose of $5\times10^4$ r; (4) DNA irradiated with dose of $3\times10^5$ r; (5) DNA irradiated with a dose of $6\times10^5$ r.

We succeeded in obtaining a monolayer of DNA by another method — by spreading dry granules on the surface of 38% ammonium sulphate solution. These experiments were carried out with dried DNA from the calf thyroid gland (Lait & Co. of England) with a molecular weight of 26,000 and a N/P ratio of 1·76 (unfortunately, our DNA of molecular weight $2\times10^6$ was heavily depolymerized on lyophilization and therefore we only had at our disposal in the form of dried granules the low-polymer form of DNA). The thickness of the layer formed, determined by the spectrophotometric method described above, was 23 Å which is in good agreement with the known data on the diameter of the DNA molecule. Hence, in our experiments a monolayer of DNA was actually obtained with the molecules lying horizontally to the interface and orientated probably by their phosphorus groups to the water.

On irradiation of 10% aqueous solution of DNA with doses from $10^4$ to $6\times10^5$ r with subsequent lyophilization, the pressure-area curves as Fig. 3 shows were virtually unchanged; the molecular weight of this low-polymer DNA was also unchanged on irradiation.

## DISCUSSION

Molecules of nucleic acids contain in all their constituent components (nitrogenous bases, carbohydrates, phosphoric acid radicals) a large number of polar groups, so that at the air-water interface they show no tendency to asymmetrical orientation. On the contrary, because of the tendency of all the polar groups to interact with the

water phase, they are arranged horizontally at the interface in like manner to polypolar compounds such as polyatomic alcohols. In fact, we found that when the molecular weight was changed by some tens of times, the thickness of the monolayers remained within the limits of 20–23 Å corresponding to the thickness of the DNA molecule, which is indicative of their horizontal position in the monolayer; only the area occupied by a given molecule in the surface layer changed.

Because of the considerable solubility of DNA in the backing solution obtaining DNA monolayers presents difficulties and as pointed out above, sometimes these layers are obtained only with the apurine form of DNA [1] containing a reduced number of polar groups. James and Mazia [2] expressed surprise that they were able to obtain a DNA monolayer with 2 M NaCl as solvent, but since they do not specify the purity of the preparation of DNA, it is possible that the results obtained by them are to be explained by the presence of a certain content of nucleoprotein in the preparation. We were able to obtain a DNA monolayer, not from solution, but by spreading solid grains on a 38% ammonium sulphate solution with a normal thickness of the monolayer of 23 Å; it is possible that by this means a monolayer of pure, chemically-unchanged (though low-polymer) DNA was obtained for the first time.

It is a comparatively easy task to obtain a monolayer of nucleoprotein since the interaction of protein and nucleic acid somewhat reduces the number of "free" polar groups and facilitates spreading, especially with reduction in solubility, by use in the backing of a concentrated solution (38%) of ammonium sulphate. It is clear from the Table that by this means it was possible to obtain monolayers of nucleoprotein with a thickness of about 20 Å for variable molecular weights, i.e. with the molecules horizontally arranged at the interface.

X-irradiation of nucleoproteins results in their profound destruction with reduction in the molecular weights (at high doses of radiation) some 200–400 times. However, the thickness of the monolayers (about 20–30 Å) and the pressure-area curves (Fig. 1) of the monolayers obtained from the native and irradiated preparations changed comparatively little. A slight shift in the pressure-area curves to the left due to solution of some of the more heavily destroyed molecules in the backing was insignificant as compared with the total destruction observed by viscosimetric measurements on the bulk solution. The slight role of partial solution of the degraded molecules also follows from the good reversibility of the pressure-area curves, even for 10 repetitions, since if there was any process of solution these curves would display a gradual shift. The findings in the Table and Fig. 1 and also the results obtained for the DNA preparation with mol.wt. = 26,000 (Fig. 3) show that even with such intense destruction the ability to form monolayers and their mechanical properties change comparatively little as compared with the high molecular weight native preparations. This result is apparently to be explained by the fact that the packing of the nucleic acid molecules and the nucleoproteins in the surface layer depends only little on the degree of their degradation. For a given lateral pressure, filling of the surface of the monolayer with the zigzags of the chain of the polynucleotide or the large fragments of this chain may be sufficiently close in density of packing with the result that the conditions of deformation (manifest

in the pressure-area curves) remain comparatively constant. It is possible also that in the biological membranes or interfaces in the presence of lateral pressure, e.g. as a result of spread of the lipoid components, a definite state of structural packing of the monolayers of the nucleoproteins may be maintained even in the presence of destructive phenomena, provided only that no chemical transverse bonds are formed in the monolayer between fragments of the chain.

We have shown that if the DNP monolayer is obtained on addition to the backing of 2% formaldehyde forming transverse lacings in the monolayer then persistent disturbances in the structure of the monolayer arise accompanied by a certain increase in the area occupied. For example, for native DNP the area increased from 0·34 to 0·43 $m^2/mg$ and for the destroyed form from 0·26 to 0·36 $m^2/mg$ (Fig. 2) and on compression of the monolayer it remained in a somewhat extended or porous state. Direct irradiation of the monolayers of DNP and DNA when the formation of "lacings" between the fragments of the polynucleotide chains is possible may also result in persistent disturbances in the structure of the monolayer leading to change in permeability and subsequent physiological disturbances in the cell. These problems must form the subject of further research.

VOL. 5 1960

## SUMMARY

(1) DNA monolayers (by the method of spreading of solid grains) and those of DNP (by the method of spreading solutions) have been obtained on a 38% aqueous solution of $(NH_4)_2SO_4$. It has been shown that the thickness of the monolayers formed for preparations of different molecular weights is 20–23 Å which corresponds to the horizontal arrangement of the molecules in the monolayer.

(2) The pressure-area curves of the monolayers obtained from native preparations and those destroyed by X-rays (doses of $10^4$–$10^6$ r) quite closely agree with each other which is indicative of uniform density of filling of the monolayer with the zigzags of the polynucleotide chain or the large fragments of this chain for a given lateral pressure.

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* There is a translation into English of this work.

# ANALYSIS OF THE EFFECT OF THE CHIEF PHYSICAL FACTORS CHANGING RADIOSENSITIVITY *

L. Kh. Eidus and Ye. E. Ganassi

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
5
196

The sensitivity of various objects to the action of ionizing radiations is determined not only by the dose, but by the influence of various agents modifying the radiation damage. Many of these agents exert an effect not only during radiation but also after it.

As investigations in recent years have shown, of increasing importance in elucidating the primary mechanism of the effect of ionizing radiation is study of the post-radiation effects. The practical importance of the search for methods of post-radiation protection also stimulates such research. At the present time, in radiobiology considerable factual material has accumulated on the effect of various modifying agents. In this review an attempt will be made on the basis of an analysis of the literature and personal data to systematize these facts from a definite standpoint.

Previously [1] we pointed to the common features observed in the manifestation of the damaging post-radiation effect of heat on irradiated living objects and biological macromolecules in model experiments, we shall call this reaction "heat" after-effect as distinct from all its other forms. It may be assumed from this common characteristic that the after-effect is due not so much to peculiarities in the metabolic processes as to the physical action of heat on changes maintained for long periods in the irradiated macromolecules.

The heat after-effect is an indication of the existence of certain changes latent in the irradiated object persisting for a long time, but leading to post-radiation damage after a definite change in the external conditions. Heat is one of the agents bringing out these latent injuries.

Another agent is oxygen. As has recently been demonstrated in model experiments with protein solutions [2] and on living objects [3–6] there exists another form of after-effect (we shall call it "oxygen" after-effect) consisting of intensification of the damage to objects on entry of oxygen after irradiation.

The distinctiveness of this "oxygen" after-effect provides further confirmation of the physico-chemical nature of the phenomenon itself and of the prolonged maintenance in the irradiated objects of latent centres of damage on which the oxygen may act.

Reference can also be made to a further series of other agents capable of modifying the radiation effect both during and after irradiation. However, oxygen and heat

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are the most universal and effective in the natural conditions of live and model objects. Therefore, we were principally concerned with discovering the nature of those centres of damage which are subject to the "attack" of these agents. In this connexion a striking aspect is the substantial parallelism in the results of a series of radiobiological investigations and the behaviour of the spectra of electron paramagnetic resonance (e.p.r.) of various irradiated living objects and model systems.

We shall start by referring to some of these parallels.

(1) In irradiated model and living objects both unpaired electrons [7–17] and the latent damage which is responsible for post-radiation reactions are preserved for a long time (hours, days).

(2) The amplitude of the resonance peaks formed on anerobic irradiation of protein preparations [7, 8], seeds [10, 16] and other objects [7–9, 13], is subsequently reduced by the effect of oxygen, i.e. in this case the "oxygen" after-effect is observed.

VOL. 5 1960

(3) According to Gordy [8, 9], the shape of the e.p.r. spectra formed on irradiation of protein preparations indicates the existence in the protein molecule's structure of two types of "traps" of unpaired electrons. Some are subject to the effect of oxygen, others are not sensitive to it and the e.p.r. spectrum produced by them is maintained for a long time even in aerobic conditions. A similar conclusion has been reached by other workers who have investigated the e.p.r. spectra of irradiated seeds [13, 16].

In experiments on irradiation inactivation of myosin solutions, fully in line with the above results, we have demonstrated [18] the maintenance in the same molecule of two different types of latent damage, one subject to the effect of oxygen, the other to the effect of heat. Similar findings [19] have been obtained in experiments on living objects (on irradiation of barley seeds — according to the criterion of chromosome aberrations).

(4) It has been established [13] that depression of the growth of plant seedlings is proportional to the number of unpaired electrons contained in the irradiated seeds before soaking them. The number of these electrons may be artificially reduced in various ways. Thus, nitric oxide sharply reduces the intensity of the e.p.r. spectrum [14] and it turns out that this is accompanied by protection as judged by biological criteria (see below). Recently, the ability of the widely known protective substance β-mercaptoethylamine to "remove" the e.p.r. signal in irradiated yeast was discovered. The reduction in the signal was proportional to the concentration of the protective substance in the irradiated suspension [20].

Thus, there is every reason to believe that unpaired electrons detected by the e.p.r. method (this method does not permit differentiation of free radicals from unpaired electrons of other types, in particular, from electrons in energy "traps") and maintained in definite conditions for a long time in the irradiated objects are directly related to radiation damage both in model and living systems. It is therefore natural to analyse those experiments in which the pathways of possible effects on these unpaired electrons are revealed. It must first be pointed out that their number diminishes under the influence of several factors (oxygen, heat, moisture, nitric oxide, etc.). This has been discovered in recent years and has still not received sufficient study. In the majority

of experiments carried out along these lines, the irradiated object was exposed to the simultaneous action of several agents, some of which competed with others or, on the contrary, sensitized their effect. This greatly confuses the picture. Thus, for example, for a correct interpretation of the mechanism of action of oxygen it is necessary to analyse the effect of moisture, nitric oxide and other agents and for an understanding of the heat mechanism, the influence of moisture, oxygen, etc.

Failure to do this leads to apparent contradictions in the effect of the same agents. Thus, for example, moisture, heat and nitric oxide in some experiments exert a protective action, in others, lead to damage.

On the other hand, as will be shown below, the reduction in the amplitude of the resonance e.p.r. peak under the influence of a particular agent may be accompanied both by a protective and a damaging effect, and only by comparing these results with the biological effect can correct conclusions be drawn.

An attempt is made in the present paper to evolve an inherently consistent scheme of influence of these factors, above all, oxygen and heat, by means of an analysis of the available literature and our personal findings.

Analysis of the work done by the e.p.r. method gives the following picture.

On irradiation, there are formed in the object unpaired electrons the number of which rises linearly with the radiation dose. In the real object with a given moisture content, the subsequent fate of these electrons will be as follows. Some of them rapidly (in the majority of cases during irradiation) vanish, and the rate of disappearance is proportional to the square of their number. We shall call this the first phase of change in the e.p.r. spectrum. As a result of this process the amplitude of the resonance peak even when measured "immediately" after irradiation gives an exaggeratedly low number of unpaired electrons as compared with the actual number formed during irradiation. This difference is greater, the greater the moisture content of the object.

This is followed by the second phase — the slow (in some conditions hours or days) reduction in the resonance peak at first rapidly, then more retarded. The rate of this reduction also depends on the moisture content. If the objects are sufficiently dry the e.p.r. spectrum in this second phase does not change even in the presence of oxygen. With a rise in the moisture content the change in e.p.r. spectrum under the action of oxygen is accelerated.

Finally, the third group of electrons remaining after the slow decrease in the resonance peak is maintained in the object for a still longer time and does not even change under the action of oxygen at increased oxygen pressure and greater moisture content (third phase).

This scheme, in our view, fits the results of all the studies quoted above, apparent deviations from it may be attributed to failure to allow for particular factors, since in each of the papers the effect of individual agents was studied and not their totality.

Thus, on irradiation of "dry" proteins and other objects in the work of Gordy *et al.* [7–9] and "dried" seeds in the work of Conger and Randolph [16] an "oxygen" after-effect was observed, manifest in a decrease in the intensity of the e.p.r. spectra. At the same time, in other studies [13–15], with sufficient dryness of similar objects

VOL
5
196

the e.p.r. signal on introduction of oxygen after irradiation was maintained without change.

This apparent contradiction can be resolved if we bear in mind that in the work [7–9] no control was exercized over the moisture of the preparations and comparison of the different findings given in paper [16] provides grounds for assuming that in the series of experiments with the "oxygen" effect the preparations designated by the authors as "dry" in reality were still not properly dried and contained sufficient water to explain the small "oxygen" after-effect.

We shall consider the findings of radiobiological investigations in which the effect of various agents modifying the damage in model and living systems was studied.

*I. Effect of the specimen's moisture content and the mechanism of "oxygen effect".* It has recently been discovered that water in particular conditions exerts a protective action.

Thus, on a range of various objects — seeds [3, 13, 14, 21] and pollen [22] of plants, bacteria [23, 24], the eggs of Artemia [25], etc. (see [11]) — increase in the moisture content of up to 12 to 16–20 per cent (varying in different investigations) on irradiation sharply increased the resistance to ionizing radiations and ultra-violet [23]. This phenomenon is observed not only in living objects but in model experiments. Recently, it was found that water protects against inactivation of DNA-ase [26]. With an increase in moisture, degradation of the hydrophil colloids of amidase and amylopectin [11, 13] and also DNA [23] is attenuated.

With an increase in the moisture content above the 12–20 per cent indicated the resistance falls so that the relation between resistance and moisture takes the form of a curve passing through a maximum. Thus, whether the increase in moisture will exert a protective or damaging effect will depend in these experiments on range of change of the moisture content. At the same time, water may intensify the damage even for low values of moisture content. This is the position on storage of seeds after irradiation [27–29]. The higher the moisture content of the seeds the more severe the damage detected on subsequent germination.

If we compare all these findings with the results given above of the work done by the e.p.r. method, the following important conclusions can be drawn and certain assumptions made.

(1) The appearance on irradiation and the presence in the object of unpaired electrons still does not lead to damage and may be regarded merely as a potential (latent) cause of possible subsequent damage.

This follows from the fact that the degree of biological damage is proportional not to the number of unpaired electrons formed by a given radiation dose but is determined by a smaller number — the number of electrons remaining after the first phase.

(2) The experimentally observed protective role of water with increase in moisture content in the irradiated objects from 3–4 to 12–20 per cent is accounted for by the fact that the water contributes to a rapid fall in the number of unpaired electrons formed in the object (first phase of changes in e.p.r. spectra).

(3) Since oxygen in biological and model systems causes only a damaging and not a protective effect, it may be assumed that the reduction in the resonance absorption by oxygen (second phase) also leads to damage and not to protection.

(4) Since in the absence of moisture, oxygen does not affect the e.p.r. spectra of the irradiated objects and the degree of radiation damage is related to their intensity, it is natural to suppose that in biological experiments also the damaging effect of oxygen can come about only by a mechanism involving participation of water.

(5) In real objects with a certain moisture content the various processes determining disappearance of the unpaired electrons in the first and second phases compete amongst themselves and therefore, the ultimate effect of oxygen may depend on the conditions of this competition, in particular, ease of access of oxygen to the latent centres of damage, the rapidity of its diffusion, the configuration of aqueous film at the surface of the molecules, etc.

These postulates above make it possible to understand certain general principles in the effects of radiation observed experimentally.

First of all, we shall consider the conditions in which the "oxygen effect" is manifest in model experiments and in living systems amenable to drying. According to a number of workers, the "oxygen effect" in such systems is observed only on irradiation of the dried specimens and is absent on irradiation of their solutions. Thus, oxygen intensifies damage on irradiation of dried preparations of trypsin [30, 31], ribonuclease [31], lysozyme [32], bacterial spores [33–35], the transforming factor [36] and cellulose [37]. On radiation inactivation of pepsin [38], trypsin [39], ribunuclease [40, 41] carboxypeptidase [42], lysozyme [32] and the transforming factor [43] in solution no "oxygen effect" is detected.

An interesting exception is myosin, which in solution is inactivated quite differently in anerobic conditions and in air. Some of the damage is due to the "oxygen" after-effect, and the rest to the direct presence of oxygen in the medium during irradiation. The "oxygen effect" is manifest in like manner in live cells where part of the damage is also accounted for by the "oxygen" after-effect.

On the basis of the scheme given above the principles of the "oxygen effect" in solutions may be explained in two ways.

A. After the completion of the first phase of change in the e.p.r. spectrum, in all the protein molecules in solution a certain number of unpaired electrons is maintained capable of interacting with oxygen. In this case the absence of an "oxygen effect" is merely apparent and due to failure to allow for existence of the phenomenon of the "oxygen" after-effect. In fact, in all the papers listed above, the authors compared the value of the inactivating effect of irradiation in anaerobic and aerobic conditions during irradiation, determination of the activity was made after irradiation with oxygen already present. If there is an "oxygen" after-effect then a difference between irradiation in the presence of oxygen and without it in such conditions could not be detected. Support for this assumption is provided by the fact that in all these experiments concordant results of anaerobic and aerobic irradiation were observed against a back-

ground of appreciable inactivation in both cases and not against a background of absence of damage to the object.

Unlike the other small protein molecules investigated, in myosin the "oxygen effect" is increased if oxygen is present during irradiation. This may be connected with a retarded course of the processes of the first phase in large molecules during which the oxygen may act on the unpaired electrons which vanish as a result of these processes.

B. On irradiation, small molecules in solution after the first phase there remain very few electrons capable of interacting with oxygen so that the latter cannot induce damage. In large molecules, such as myosin, they are sufficiently numerous even in solution. From this point of view the large molecules should display an after-effect to a high degree and in greater measure exert an influence on the level of the after-effect in living cells. This second explanation, though possible, still seems to us the less probable, since we have experimentally demonstrated that in macromolecules even on irradiation in solution, a considerable number of unpaired electrons is preserved (see below).

VOL. 5 1960

With regard to the "oxygen effect" in so-called "dried" objects, the sole explanation of this phenomenon is the insufficient degree of desiccation of the object. In fact, in those cases in which the dryness of the object was adequate the "oxygen effect" in the normal sense of the term was not observed. Thus, in the experiments of Ehrenberg [14] damage to seeds with a moisture content of $< 4$ per cent was uniform on irradiation in nitrogen and in oxygen (the damage itself here was due apparently to an "oxygen" after-effect during the soaking of the seeds).

A similar result was observed in the experiments of Shapiro and Bocharova (verbal communication) in which damage to dry barley seeds (4–5 per cent moisture) was compared on irradiation *in vacuo* and in air. The damage (number of chromosome aberrations) was in both cases due to an "oxygen" after-effect and did not depend on the conditions of irradiation.

The absolute value of the degree of dryness sufficient to remove the "oxygen effect" obviously differs for different objects, in particular, seeds and finely-dispersed protein preparations.

It must be assumed that in the experiments enumerated above with "dried" objects the degree of dryness, though high enough to reduce the processes of the first phase (which corresponded to the "oxygen effect") at the same time was insufficient to prevent complete development of the second phase in which oxygen exerted an injurious effect. As stated above, a similar situation has been observed also in the investigation of the e.p.r. spectra [14–16].

*II. Effect of heat.* If we are to go by the data in the literature, rise in the temperature of the medium during or after irradiation may exert, in the same way as a variable moisture content, both a damaging and protective effect. A protective effect of heat in the interval from freezing point to room and higher temperatures has been observed for various objects and for different criteria of radiosensitivity: on irradiation of seeds — according to germination and growth of seedlings [21, 27, 44–47], chromosome aber-

rations [27, 48]; on irradiation of *Drosophila* [49, 50] and their eggs [51, 53] and also the microspores of *Tradescantia* [54–56] according to chromosome aberrations and, finally, on ultra-violet and X-irradiation of bacteria — according to their survival rate [57, 58]. In addition, there are a few papers [59–61] in which the temperature dependence is complex (protection alternates with damage) so that the phenomenon becomes very involved and does not easily lend itself to interpretation.

On analysis of the conditions for the manifestation of the protective effect of heat in the studies listed above, the following general principles are apparent. A protective effect is observed on heating the object not only during but immediately after irradiation. On irradiation of seeds the protection by heat is greater, the higher the moisture content [27, 28] and in sufficiently dry seeds temperature, in general, has no effect [21]. On neutron irradiation the effectiveness of which does not depend on the moisture of the objects [11, 27, 29], temperature protection is also not observed [27].

It follows from this that the protective effect of heat comes about only in the presence of water. In accordance with the ideas considered in the preceding section, it may be assumed that the protective effect of heat is due to one of two phenomena (or both together): (1) intensification of the processes of the first phase; and (2) reduction with rise in temperature of the solubility of oxygen in the object. Intensification of the process of disappearance of unpaired electrons (acceleration or intensification) in the first phase must cause attenuation of all the after-effect reactions and, in particular, make it less possible for oxygen to cause damage. The recent calculations of Evans [62] showed that the protective influence of raised temperature on irradiation of seeds may be wholly explained by the reduction in concentration in them of dissolved oxygen. Apparently, this explanation is also applicable to the other cases since use was made in them of live objects containing a definite amount of moisture.

Thus, in our view, the protective effect of heat comes about in one way or another by change in the effectiveness of the oxygen action on the irradiated objects.

The damaging effect of heat is realized in a quite different way and, unlike the protective effect, is not associated with oxygen. This is indicated in the first place by the experiments mentioned previously which showed that the centres of latent damage subject to the attack of oxygen and heat vary. We demonstrated this in model experiments with protein solutions in which the criterion of radiosensitivity was loss by the protein of its enzymatic activity [18]. In the solution of the irradiated myosin both forms of after-effect were observed. By means of consecutive heat and oxygen treatment the independence of the influence of these agents was demonstrated and as a result the existence of at least two types of latent damage.

A similar result was given by experiments on the live objects — irradiated barley seeds subjected to heat and oxygen treatment during soaking [19].

Thus, oxygen and heat act on different centres of damage. However, the effect of both agents leads to reduction in the number of unpaired electrons maintained in the irradiated objects. Attenuation of the intensity of the e.p.r. spectra under the influence of oxygen is quite well known [7–10, 13, 16].

One of us previously put forward the hypothesis [1] that the "heat" after-effect widely observed in model and living systems is due to the action of heat on unpaired electrons maintained for a long time in biological macromolecules. Recently, we confirmed this experimentally [63]. In these experiments, myosin (10 mg protein per 1 ml 0·5 M KCl) and pepsin (5 mg protein in 1 ml 0·2 M acetate buffer) solutions after irradiation in air with $\gamma$-rays were dried and unpaired electrons were detected by the e.p.r. method in the dry preparations. If before drying the irradiated solutions were subjected to heat treatment in conditions in which the "heat" after-effect was manifest to completion (20°, 24 hr for myosin; 54°, 6 hr for pepsin) unpaired electrons could no longer be detected.

This confirms the assumption of the rôle of unpaired electrons maintained in the object and not subject to the effect of oxygen (third phase of change in the e.p.r. spectrum) in the phenomenon of the "heat" after-effect.

VOL. 5 1960

The analysis made by us shows that the damaging effect of oxygen comes about only in the presence of moisture. It is shown that the damage by heat is characterized by the same distinctive feature. This may be illustrated by experiments carried out by us [64] with dried preparations of pepsin irradiated with $\gamma$-rays. Heat inactivation of such preparations between 100 and 130° was of an exponential form and occurred at all temperatures at an equal rate in the irradiated and control samples. No irradiation after-effect was detected in these conditions. When, after incubation in the dried form the same preparations were dissolved and submitted to heat inactivation in solution (54°, 6 hr) they showed a marked after-effect in the same way as after irradiation in solution.

Thus, in the absence of moisture in the dried objects, heat can neither remove the cause of the heat after-effect nor "reveal" it. Taken as a whole these findings indicate prolonged maintenance in the irradiated objects of "heat" centres of latent damage subjected to "attack" by heat only in the presence of moisture.

This also offers an explanation for such widely-known phenomena as increase of damage with rise in moisture to irradiated seeds on storage [27–29] and the absence of heat effect in dried seeds [21]. In this, we have all the features characteristic of "heat" and "oxygen" after-effects.

*III. Effect of nitric oxide.* In recent years, in a number of laboratories, investigations have been made of the effect of nitric oxide on the radiosensitivity of certain objects [14, 33, 34, 65–69] and on the intensity of the corresponding e.p.r. spectra [14]. The use of nitric oxide in connexion with attempts to elucidate the physical nature of the mechanism of the action of oxygen in radiobiological investigations is logical. Both these gases are paramagnetic; in the unexcited state they constitute radicals; in fluorescence phenomena, they possess many similar properties as quenching substances, etc. In fact, in a number of radiobiological experiments it has been shown that nitric oxide has the same damaging effect as oxygen and in conditions of anoxia can replicate it on a 1:1 basis (on irradiation of yeasts, [65], *Vicia faba* [60], bacteria [65, 67] and Ehrlich ascites carcinoma cells [68]). This once again emphasizes that the damaging effect of oxygen in irradiation is due to physical and not metabolic

causes. However, recently it has been discovered that the damaging effect of nitric oxide is observed only in the presence of a sufficiently high moisture content. Where the moisture content is less (4–12 per cent) the effect of both gases greatly differs: introduction of nitric oxide raises the resistance after irradiation both in oxygen and in nitrogen [14], the difference between the gases being greater, the lower the moisture content of the object. The most interesting point is that in this moisture content range, nitric oxide exerts just as marked an effect on the e.p.r. spectra of these objects. Unlike oxygen, nitric oxide "removes" the resonance absorption even in dried preparations, i.e. conditions in which oxygen is inert in this respect. Such a reduction in the intensity of the e.p.r. spectrum by nitric oxide can be produced a long time (several days) after irradiation.

It obviously follows from all this that reduction in the intensity of the e.p.r. spectrum by the interaction of nitric oxide and unpaired electrons leads to protection of the objects.

The experimental findings available in the literature on the use of nitric oxide are still insufficient for a definitive judgement of the details of its mechanism of interaction with these electrons. However, it is noteworthy that the damaging effect both of nitric oxide and oxygen is brought about only with the participation of water.

VOL
5
196

## SUMMARY

Comparison of the results of experiments on irradiation of biological objects *in vivo* and *in vitro* with the data obtained by the e.p.r. method leads to the following conclusions.

On irradiation, unpaired electrons subject to the action of a number of agents are formed in macromolecules and in biological objects and may be maintained for a long time. The subsequent fate of these unpaired electrons varies.

A part of them vanish in the presence of sufficient moisture without producing damage. The protective rôle of water is connected with this.

The effect of oxygen on the second part produces damage ("oxygen effect"), which only comes about in the presence of water.

Damage by heat ("heat" after-effect) is associated with the action on the third part of the unpaired electrons and this damage is also only possible in the presence of water. The protective rôle of heat observed in a number of experiments is due to change in the ratio of the effects of the various agents modifying damage.

The fact that the damaging effect of all three agents considered — heat, oxygen and nitric oxide — comes about only with the participation of water is worthy of attention.

In our experiments with protein solutions disappearance of unpaired electrons under the influence of the modifying agents was accompanied by irreversible loss of enzymatic activity. Since in the general case the region of localization of unpaired electrons apparently is away from the enzymatic centres, these processes must incorporate migration of the charge and energy.

The presence of water turns out to be just as necessary for this as is the effect of the modifying agents themselves.

It must be borne in mind that water in this case may act as a substance through which migration occurs and as a factor stabilizing the structure of the macromolecules and thereby creating the possibility for migration over the structure itself.

Consideration of the physical mechanism of action of the agents modifying radiation damage does not come within the framework of the present review. We would merely point out that the possible mechanism of their effect on the unpaired electrons maintained in macromolecules and on the protein structure itself, in connexion with its water of hydration, can be regarded on the same footing.

VOL. 5 1960

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* There is a translation into English of this work.

VOL
5
196

# CHANGE IN RADIOSENSITIVITY OF ERYTHROCYTES*

A. M. KUZIN and K. S. TRINCHER

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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THERE are two fundamental cytophysiological aspects of the problem of the radiosensitivity of a living system: first, the nature of the initial mechanism of action of penetrating radiations on living matter and, secondly, the minimum dose of radiation to which the living system reacts. Solution of these problems requires first of all a clear definition of the basic concept of radiobiology — the concept of the radiosensitivity of the cell. We suggest the following definition.

The radiosensitivity (r.s.) of the cell is a value inversely proportional to the minimum dose of penetrating radiation producing irreversible damage to the morphophysiological structure of the living system.

We propose that all other reactions not giving irreversible after-effects and having the character of reversible changes should be incorporated in the concept of radiolability of the cell.

Radiolability (r.l.) of the cell is a value inversely proportional to the minimum dose of penetrating radiation producing reversible changes in the morphophysiological structure of the living system.

The difference between cells of high radiosensitivity, e.g. bone marrow cells (I) and of low radiosensitivity, e.g. cells of the nervous system (II) is expressed in the following manner:

$$\text{r.s.}_{\text{I}} > \text{r.s.}_{\text{II}}$$

while at the same time the ratio

$$\text{r.l.}_{\text{II}} \gg \text{r.s.}_{\text{II}}$$

applies, i.e. the radiolability of nerve cells — their ability to react reversibly to the radiation stimulus — considerably exceeds their radiosensitivity.

In the present investigation it will be shown that cells possessing low radiosensitivity in natural conditions (erythrocytes) are transformed by certain changes in their environment into cells with high radiosensitivity. Analysis of the experimental data enables us to put forward theoretical arguments on the mechanism of radiation damage to the cell for limiting small doses.

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## EXPERIMENTAL

Fresh rat blood was used as test object. The criterion of irreversible radiation damage to the red cells for a dose $D$ was the shortening in time of onset of haemolysis in isotonic alkaline buffer $t_{hem}$ $(D)$ as compared with the controls, $D = 0$. As was shown in a previous paper [1] at low doses

$$t_{hem}(D) \approx \frac{m_0}{k_2} e^{-k_1 D}, \tag{1}$$

where the constant $m_0$, the undamaged mass in the surface layer of the cell, $k_2$, the rate of solution of this mass in the buffer and the constant $k_1$, the sensitive volume ($\sim 0{\cdot}83 \times 10^{-17}$ ml) in the surface layer of the cell. From (1) it follows that the difference between the times of onset of haemolysis for the unirradiated and irradiated blood is equal to

$$\Delta t = \frac{m_0}{k_2}(1 - e^{-k_1 D}). \tag{2}$$

From (2) it is clear that the radiation damage to the erythrocytes may be detected theoretically for infinitely small doses ($D \to 0$) if simultaneous with an decreased dose there is corresponding reduction in the parameter $k_2$, i.e. for

$$D \to 0 \text{ and } k_2 \to 0, \text{ then } \Delta t = \text{const.}$$

Since $k_2$, the other conditions being constant, depends solely on temperature and concentration of the OH ions, it follows that lowering either the temperature or reducing the pH of the medium separately or together can theoretically indefinitely extend the sensitivity of the given method. We tested this thesis experimentally.

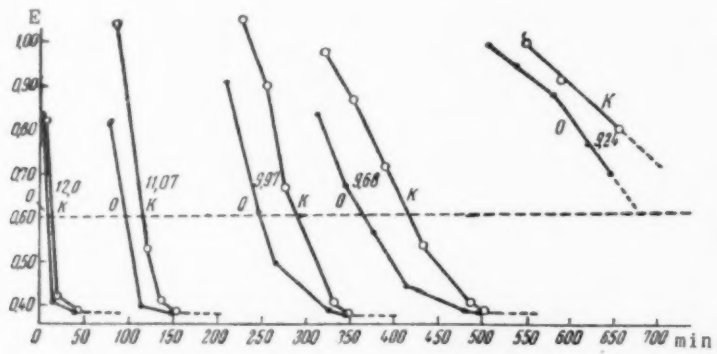


FIG. 1. Effect of pH of buffer on detection of radiation damage to red cells ($\Delta t$) at a dose of $3 \times 10^3$ r. ($O$, experimental; $K$, controls; $t = 32°$).

The experiment was carried out with a red cell suspension (dilution of fresh blood in saline 1:200) irradiated with $\gamma$-rays from a cobalt unit ($\sim$ 200 r/min). Immediately after irradiation, we added 1 ml of the suspension to 5 ml of buffer solutions of varying

pH and incubated then at $32 \pm 0{\cdot}2°$. After definite time intervals, the extinction of the irradiated samples ($O$) and the corresponding unirradiated controls ($K$) was measured on a photoelectric colorimeter (FEK-M). Figure 1 gives the results of one experiment with a dose of $3 \times 10^3$ r. The figures against curves $O$ and $K$ denote the pH of the buffer solution.

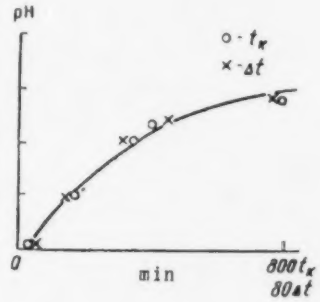


FIG. 2. Variation of $\Delta t$ and time of onset of haemolysis of controls $t_k$ with pH of solution. Ordinates, values of pH in descending order from 12, 11, 10, 9 and 8.

Each curve — extinction ($E$) as a function of time — has an initial portion with a high value of $E$ (turbid solution with individual cells), then follows a sharp drop in extinction — the actual process of haemolysis of the entire cell population — and, finally, the minimum value for $E$ of the haemolysed solution, unchanged in time. Similar curves obtained in a total of 20 repeat experiments differed from each other

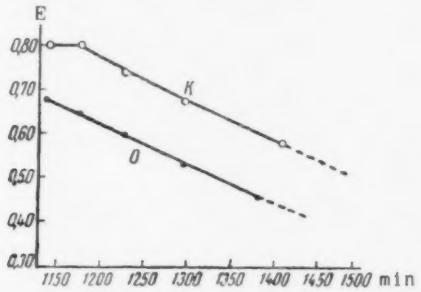


FIG. 3. Haemolysis (change of extinction with time) at 0° and pH 11·07. ($O$, experimental — dose $3 \times 10^3$ r; $K$, controls).

by not more than 5–8 per cent for a uniform concentration of red cells. Higher cell concentrations had higher value of minimum extinction, the curves were shifted to the right, — the haemolytic process occurred later. From the experimental curves in Fig. 1 we can obtain a graphic representation of the value $\Delta t$ as a function of pH and also of the time of onset of haemolysis of the control samples (the value $t_k$) as a function of pH. Figure 2 depicts the relation between $\Delta t$, $t_k$ and pH; the values

of $\Delta t$ and $t_k$ are superimposed by adjusting the scale on the abscissa. The experimental points correspond to their values for an extinction of 0·60 (the values at pH 9·24 were obtained by extrapolation).

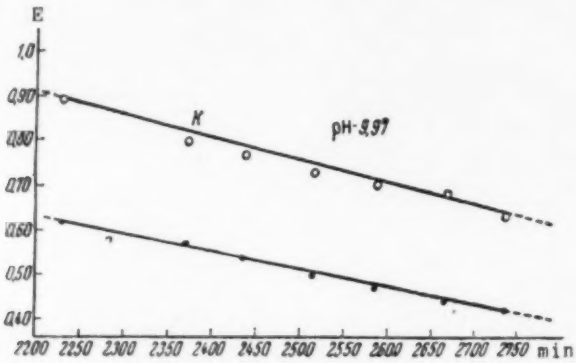


FIG. 4. Haemolysis (change in extinction with time) at 0° and pH 9·97. (*O*, experimental — dose $3 \times 10^3$ r; *K*, controls).

As Fig. 2 shows $\Delta t$ and $t_k$ rise with decrease in the concentration of OH ions (reduction in the parameter $k_2$ in equations (1) and (2)). The second effect predicted by theory — increase in $\Delta t$ with a reduction in temperature — is depicted in Fig. 3 and 4 for pH 11·07 and 9·97 at 0° and for a radiation dose of $3 \times 10^3$ r.

As Fig. 3 and 4 show, the sensitivity of the method is sharply raised when haemolysis in alkaline medium is retarded by a reduction in temperature and a decrease in the OH ion concentration.

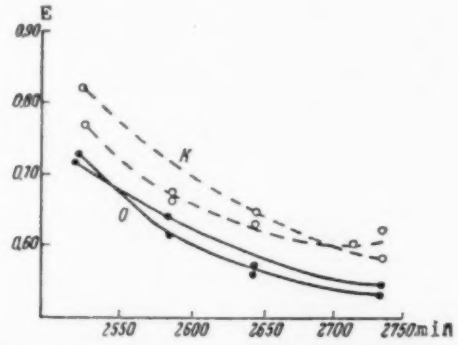


FIG. 5. Haemolysis (change in extinction with time) at 0° and pH 9·97. (*O*, experimental — dose 500 r; *K*, controls).

Figure 5 gives the experimental data obtained at 0° and pH 9·97 when the red cell suspension was irradiated with γ-rays to a dose of 500 r. The minimum dose producing radiation damage to the red cells detected by this method corresponds to the data

of the sole work in which it was possible to detect radiation damage to red cells produced by a dose of 400 r [2].

The red cells suspended in saline (dilution of blood 1:200) are consequently very sensitive cells, radiation damage to which may be detected by the quite sensitive method of alkaline haemolysis at 0° after irradiation with 500 r of γ-rays. The question arises as to whether the high radiosensitivity of the red cells detected *in vitro* is dependent on the state of the extracellular medium. In order to explain the effect of the extracellular medium on the red cells, two series of experiments were performed: on the

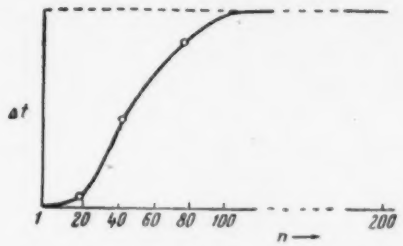


FIG. 6. Radiation damage to red cells ($\Delta t$) as a function of degree ($n$) of dilution of serum by saline.

animal and in *vitro*. The results of these experiments show that irradiation of rats with $3\times10^3$ r, γ-rays does not cause radiation damage to the red cells which is detectable by the method of alkaline haemolysis. The *in vitro* experiments gave the same results; a suspension of red cells in serum, diluted up to 10 times, was completely protected from radiation damage by a dose of $3\times10^3$ r of γ-rays; the protective effect fell off for higher dilutions. Figure 6 shows the relation between $\Delta t$ and the dilution of serum ($n = 1, 2, 3....$) in saline for a dose of $3\times10^3$ r and pH 9·97.

It follows from these findings that in natural conditions red cells are highly radioresistant, owing to the protective effect of the blood plasma. With a sharp reduction in the serum proteins *in vitro* (diluted 200 times) the red cells become radiosensitive, exhibiting radiation damage after a dose of 500 r of γ-rays.

## DISCUSSION

It follows from the experiments described here that in an artificial medium not containing mobile protein molecules, red cells are transformed from radioresistant to radiosensitive cells. Absence of mobile protein molecules in the intercellular solution deprives the structural protein complexes of the surface layer of the red cells of their natural defence against radiation damage. Since in natural conditions in unicellular organisms and in the dividing cells of the multicellular organism, phases of variable radiosensitivity are observed, the hypothesis may be advanced that in natural conditions also replacement of the radioresistance of the cell by radiosensitivity may occur — associated with the appearance of an aqueous solution in the cell nucleus containing little or no protein. The plausibility of this hypothesis is based on the following phenomena in the physiology of cell division [3].

(1) The highest radiosensitivity of dividing cells is observed at prophase when the volume of the cell nucleus is greatly increased and when the volume of the entire cell has already reached its maximum.

(2) The mitotic apparatus has a lower refractive index (consequently contains more water) than the cytoplasm.

(3) Increase in the volume of the cell nucleus results in gelatinization of the cytoplasm which may be connected with the previous passage of water from the cytoplasm into the cell nucleus.

The cytophysiological data presented indicate that expansion of the cell nucleus is associated, apparently, with entry of liquid with a relatively low content of mobile protein molecules into it. We consequently have at prophase a situation similar to that in which in artificial conditions *in vitro* there is an increased radiosensitivity of the red cells: the presence of a non-proteinous solution in contact with the structural protein complexes of the surface layer of the red cells. As with the red cell in artificial conditions the dividing cell at prophase acquires radiosensitivity when the structured protein complexes of the cell nucleus are surrounded by an aqueous medium with a low concentration of mobile protein molecules.

Our experiments show that the radiation damage to the cell surface of the erythrocytes is caused in the main by the radicals formed by radiolysis of water. This conclusion follows from the fact that red cells on irradiation with a dose of $3\times10^3$ r *in vivo* or in a protein solution *in vitro* were not damaged when the effect of irradiation was determined by the method of alkaline haemolysis. Mobile protein molecules adsorbed on the structural protein complexes of the surface layer of the red cells gave apparently complete protection both against the radicals and the direct effect of irradiation. However, irradiation with a much smaller dose (500 r) caused radiation damage to the cells, as detected by the method of alkaline haemolysis, if the red cells were suspended in a non-proteinous solution. The radicals formed on radiolysis of the water surrounding the cell produced damage to the cell surface in the absence of protective proteins. Taking the mean free path of the radical in water to be $50\times10^{-8}$ cm [4] and the surface of the erythrocyte as $\sim 200\times10^{-8}$ cm$^2$ [5] we can compute the number of radicals formed around the cell and entering it for a dose of 500 r:

$\sim \frac{1}{6}\ (500\times1{\cdot}6\times10^{12}\times50\times10^{-8}\times200\times10^{-8}) \approx 130$ radicals ($1{\cdot}6\times10^{12}$, number of ion pairs formed per ml water per r).

According to this calculation for 500 r, the cell surface of the red cell in a non-protein solution is subject to damage from the effect of $\sim$ 130 radicals formed in the water surrounding the cell. It is possible, in principle, that only one radical hitting the red cell could cause irreversible damage which could be detected by a sufficiently-sensitive method. According to this calculation one radical corresponds to a dose of $\sim$ 4 r ($500/130 \approx 4$). A dose of 4 r therefore signifies the minimum dose at which equal damage occurs to all the red cells of the cell population; at a dose of $<4$ r radiation damage in the red cell population should display statistical regularity.

SUMMARY

(1) A method has been evolved for detecting radiation damage to red cells suspended in saline for a dose of 500 r γ-rays.

(2) The protein molecules of the blood plasma exert a protective effect against penetrating radiations on the structural protein complexes of the surface layer of the red cell. This protective action was detected at doses up to $3\times10^3$ r.

(3) A hypothesis is advanced on the radical mechanism of radiation damage to the cell at limiting low doses of penetrating radiations.

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* There is a translation into English of this work.

VOL. 5 1960

# CHANGE IN THE PROPERTIES OF ERYTHROCYTES WHEN EXPOSED TO SUB-HAEMOLYTIC DOSES OF GAMMA-RADIATION *

IU. A. KRIGER and I. M. PARKHOMENKO

Lomonosov State University, Moscow

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THE traditional concepts of the exceptional radioresistance of anuclear erythrocytes of mammals are based, as a rule, on studies of radiation haemolysis. This usually occurs under the action of very high doses of radiation (of the order of hundreds of kiloroentgens) and at some time after irradiation [1–3]. However, these periods may be considerably shortened provided the erythrocytes are immediately incubated after the action of the ionizing radiation [4].

Further investigations have shown, however, that irradiation of erythrocytes with relatively low doses, not producing haemolysis, results in a number of fundamental

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changes in their physicochemical properties. Such changes include passage of cations from the irradiated erythrocytes [5, 6] and increase in their volume [7], etc.

One of the reliable indications of disturbances in the normal state of erythrocytes after irradiation is a change in the reaction of these cells to other stimuli; the influence of hypotonicity and heating [8], dilute solutions of acids [9] and bases [10]. In the work of one of the authors of the present article, it was shown that on treating erythrocytes irradiated with a dose of 80 kr γ-rays but not subjected to incubation, with distilled water, their low-frequency resistance did not rise (as normally happens) but, on the contrary, slowly and steadily fell. Further investigations carried out by the method of electron microscopy also revealed in erythrocytes, irradiated and then subjected to hypotonic haemolysis, the presence of craters and holes absent in the unirradiated control cells [4].

On the basis of these findings we sought to ascertain whether relatively low doses of radiation (a few kr) are capable of changing the sensitivity of erythrocytes to other damaging factors, using for this purpose photodynamic action. Choice of this factor was not fortuitous. It was determined by the known similarity in the primary physicochemical processes underlying the action of ionizing radiation and photodynamic action — a similarity connected with the formation in both cases of short-lived free radicals.

## EXPERIMENTAL

In the experiments a 1% suspension of rat erythrocytes, washed 3 times and suspended in saline was subjected to the action of $^{60}Co$ γ-rays from a GUT-400 unit. The radiation dose was 10 kr at a dose rate of 400 r/min. A suspension of unirradiated erythrocytes served as control. Immediately, and also 3 and 24 hr after irradiation, the suspension of erythrocytes was diluted with 0·04% erythrosine solution and irradiated for 10 min with visible light. The source of illumination was a 500 W incandescent lamp, 25 cm distant from the cuvette containing the suspension. In order to eliminate the long-wave thermal rays a filter prepared from 0·5% copper sulphate solution was used.

The course of photodynamic haemolysis was studied by measuring the light transmission of the suspension on a FEK-52 photoelectrocolorimeter, using a No. 8 red filter.

As the results showed, the rate of photodynamic haemolysis of the previously irradiated erythrocytes was always appreciably greater than that of the controls not treated with γ-rays (Fig. 1).

Consistent results were given in 94–96 per cent of the parallel experiments.

Hence, preliminary γ-irradiation undoubtedly raises the sensitivity of erythrocytes to photodynamic action. However, the reasons for the rise in sensitivity to the photodynamic action of the irradiated erythrocytes remained unclear to us.

A possible explanation may be the intensification produced by irradiation of the photosensitizing properties of haemoglobin. In order to verify this assumption, a suspension of erythrocytes irradiated with a known large dose of γ-rays (40 kr) was

subjected to the action of visible light from the same incandescent lamp. A suspension of the same irradiated erythrocytes placed in the dark served as control. As the measurements of light transmission showed, the slow haemolysis due to γ-rays (40 kr) was not accelerated under the action of visible light.

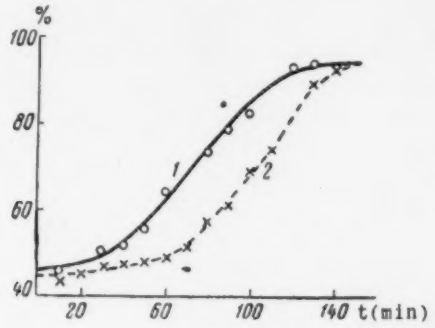


FIG. 1. Effect of preliminary γ-irradiation on photodynamic haemolysis of erythrocytes. Ordinates, light transmission, %; (1) irradiation; (2) control.

The absence of the properties of a photosensitizer in the haemoglobin treated with γ-rays was also indicated by other special experiments. In these experiments intact erythrocytes were suspended in a haemoglobin solution previously irradiated with a high dose (100 kr) of γ-rays. In preparing the latter we used a 0·1% suspension of erythrocytes washed 3 times in distilled water. The suspension was carefully shaken and left for 20 min at room temperature until the opaque blood was completely transformed into a lacquer. Then sufficient dried crystalline $Na_2SO_4$ was added to the solution to make the final salt concentration isotonic (0·11 M). The suspension was again shaken and after centrifugation the supernatant which was irradiated was drawn off. On completion, the haemoglobin solution was used as the suspending medium for the erythrocytes. The content of the latter in suspension was 0·01 per cent. The suspension was divided into two aliquots, one being left in the dark for control and the other illuminated for 40 min with visible light in the conditions described above.

As the experiments showed, haemolysis in this case was not observed either in the test or control samples. In other words, the haemoglobin solution after exposure to γ-rays does not exhibit photosensitization.

In order to further satisfy ourselves of the absence of any changes arising in the molecular structure of the haemoglobin from the action of γ-rays, we carried out spectrophotometry of the haemoglobin solutions prepared by the method described above and irradiated with doses of 10, 25 and 50 kr. In the spectrophotometric investigation, we used haemoglobin solutions prepared as above but additionally diluted (in a ratio of 1:4) after irradiation. As the results of spectrophotometry carried out in a wavelength range of 400–750 mμ with a SF-2 M spectrophotometer and an auto-

matic recorder show, no differences were observed between the absorption spectra of the control and irradiated solutions of haemoglobin (Fig. 2).

Hence, the summation of the effect of γ-rays and the photodynamic action on erythrocytes detected by us cannot apparently be explained by the appearance of the properties of a photosensitizer in the irradiated haemoglobin. It seems to us, therefore, more reasonable to explain the results obtained on the basis of the undoubted similarity in the primary mechanisms responsible for the effect of ionizing radiation on the one hand, and the photodynamic action, on the other. As is known, both processes are accompanied by formation in the aqueous phase of short-lived free

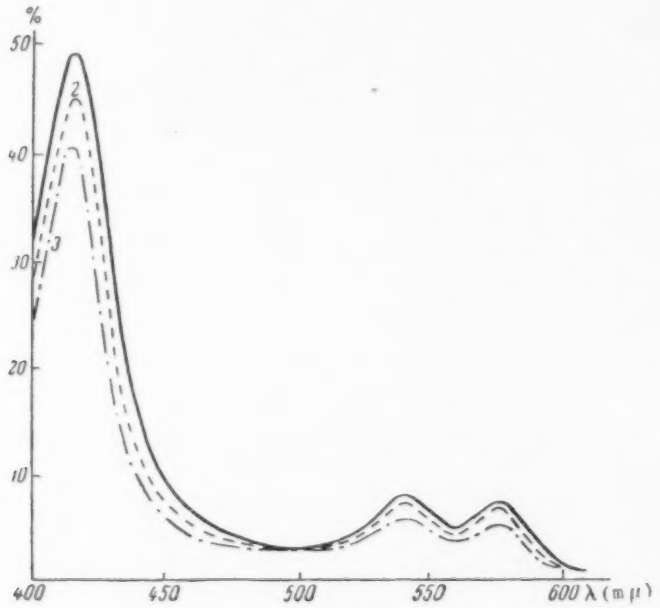


Fig. 2. Absorption spectra of haemoglobin irradiated with γ-rays. Ordinates, light transmission, % (1) 50 kr; (2) controls; (3) 25 kr.

radicals possessing the character of oxidizers. That is why summation of the effect of both factors, in our view must be based on an increase in the oxidizing effect of the aqueous solution in which the erythrocytes are immersed.

Evidence in favour of this assumption is provided by the similarity in the quantitative regularities in the protective action of thiourea previously detected by us [11] during radiation and photodynamic haemolysis.

It may be assumed from the results that radiation damage to erythrocytes must be based on the interaction of the aqueous radicals formed during irradiation and the most labile components of the stroma and, in the first place, lipoids.

To verify this supposition we applied the test of the so-called "inversion of haemolysis" (i.e. shrinkage of erythrocytes subjected to hypotonic haemolysis under the influence of solution of low molecular substances, salts and sugars). The absence of "reversion" in these conditions, according to the findings of previous studies [12], would indicate disintegration of the lipoprotein complexes forming part of the stroma.

In our experiments a 1% suspension of erythrocytes, washed 3 times in saline, was prepared. The suspension was divided into two portions, one of which served as control. The other was exposed to γ-rays, doses of 8 or 10 kr at a dose rate of 400 r/min. On termination of irradiation, half of the suspension was diluted 1:10 with saline. The other half was diluted 1:10 with distilled water and after 20 min, reversion was carried out by adding dried $Na_2SO_4$ to give an isotonic concentration of 0·11 M. The unirradiated erythrocyte suspension was treated in the same manner. Then, under the microscope a count was made in a Goriayev chamber of the intact and reverted erythrocytes in the control and irradiated samples and the percentage reversion computed. As the experiments show, irradiation as such did not change the number of erythrocytes. However, the percentage reversion of erythrocytes after irradiation and the action of distilled water, was heavily reduced as compared with the control not first subject to the action of irradiation. The percentage reversion in the control was $45 \pm 1{\cdot}6$ per cent, at a dose of 8 kr, $23 \pm 3{\cdot}7$ per cent and at a dose of 10 kr, $16{\cdot}3 \pm 3{\cdot}4$ per cent.

Hence, our assumption of disturbances in the physicochemical structure of erythrocytes when irradiated with relatively low doses of γ-rays received additional confirmation.

The findings presented in this paper permit us to interpret radiation damage to the erythrocytes as being the result of an irradiation-chemical process weakening the link between the structural components of the stroma of the red blood corpuscles.

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# REMOTE EFFECT OF RADIATION ON MOUSE TESTES*

M. D. POMERANTSEVA

Institute of Genetics, U.S.S.R. Academy of Sciences, Moscow

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INFORMATION is now available indicating that irradiation, besides inflicting direct local damage on organs and tissues, also exerts a remote effect [1–3]. However, the question of the rôle of the distant effect of radiation in damage to the gonads still remains an open one. The published findings on this problem are few in number and inconsistent. While some investigations have failed to reveal a remote effect of radiation on the testes [4] and the ovaries [5] of mammals, other workers have observed such an effect. Thus, according to Shaver [6], considerable damage to the rat testes after application of a dose of 500 r was observed only on whole-body irradiation and was absent on local irradiation of the testes. Fedorov [7] notes that on local irradiation of the rat testes with a dose of 550 r the degree of damage is somewhat less than on whole-body irradiation. After local irradiation, recovery of spermatogenesis and fertility is observed, whereas on whole-body irradiation with the same dose, even 90 days after irradiation the testes remained wasted and on testing for fertility, 6 months after irradiation, all the animals were still sterile. However, when the testes are shielded on whole-body irradiation with a dose of 550 r, they do not develop radiation injury.

The object of the present investigation was to compare the degree of damage to the testes on local and whole-body X-irradiation. The criteria of the degree of damage to the testes were changes in their weight and histological structure. The work was carried out on male mice of the A line, aged 3 months. There were three groups of animals: (1) subjected to whole-body irradiation at a dose of 400 r; (2) animals in which only the region of the testes was irradiated with the same dose; and (3) controls not subjected to irradiation.

Before irradiation the animals were tied to a wooden board, the testes brought out into the scrotum to which a ligature was applied, preventing their withdrawal. Two mice were irradiated simultaneously, one with whole-body irradiation and in the other, the entire body, except the scrotum region, was screened with lead 3 mm thick. The conditions for X-irradiation were; 15 mA, 190 kV, 0·5 Cu+0·75 Al, focal distance 50 cm, dose rate 34 r/min.

The animals were killed for weighing and histological examination of the testes on the 28th or 42nd day after irradiation. The testes were fixed in Zenker fluid (as modified by Maximov) sections 6 μ thick were prepared and stained with acid-haemalum according to Meyer. The preparations were inspected at a magnification of ×300.

* Biofizika **5**: No. 5, 543–545, 1960.

For histological analysis of the testes the technique proposed by Fogg and Cowing [8] was applied and supplemented by three-group system for evaluating the cell count in the tubule [9]. The essential feature of this technique is that from the middle of the section of testes 100 transverse sections of the seminiferous tubules are taken at random and a count is made of the various types of germinal cells in them. After evaluation of the cell content in the tubule by the three-group system, (many, average number, low) the number of tubules of each type is estimated.

It has previously been shown that irradiation with a dose of 400 r leads to considerable damage to the testes expressed in a fall in their weight and profound disturbances in spermatogenesis. The maximal fall in weight occurs by the 28th–30th day after irradiation [9, 10]. Thus, the times we chose for killing the animals enabled us to follow up the changes in the weight of the testicles, both at the moment of its greatest decline (28 days) and on recovery of weight (42 days).

OL.
5
960

TABLE 1. CHANGE IN WEIGHT OF TESTES AFTER LOCAL AND WHOLE-BODY IRRADIATION

| Type of treatment | Time after irradiation | | | | | |
|---|---|---|---|---|---|---|
| | 28 days | | | 42 days | | |
| | No. of animals | Weight of testes | | No. of animals | Weight of testes | |
| | | in mg | as % body weight | | in mg | as % body weight |
| Whole-body irradiation (400 r) | 11 | 80·4 | 0·27 | 9 | 110 | 0·43 |
| Local irradiation of testes (400 r) | 11 | 77·2 | 0·28 | 9 | 107 | 0·40 |
| Unirradiated controls | 3 | 163 | 0·62 | 3 | 196 | 0·71 |

The Table shows that the weight of testes on the 28th day after irradiation was uniform both on local and whole-body irradiation. The same was observed for recovery of the weight of the testes, i.e. on 42nd day after irradiation.

The Figure shows the result of the histological analysis of the testes. It has already been shown that irradiation leads to a gradual disappearance from the seminiferous tubules of the germinal cells starting with the most immature, i.e. spermatogonia [9]. At a dose of 400 r complete devastation in all the semeniferous tubules did not occur since in the majority, disappearance of the mature cells was accompanied by the appearance of younger forms. On the 28th day after irradiation restoration of the cells of the germinal epithelium of all, except the sperms, was observed. At this time only spermatogoniae were completely restored, whereas the other types of cells (primary spermatocytes, spermatocytes, spermatids) were represented in smaller number than in the controls. The sperms at this stage had still not completely disappeared and were contained in a few tubules, principally in the form of solitary cells.

The Figure shows that 28 days after irradiation there was no difference in cellular composition in the seminiferous tubules of animals subjected to local and whole-body irradiation. Only the percentage of tubules containing sperms was somewhat higher

in the case of local irradiation than on whole-body irradiation. However, the difference was not statistically significant and apparently, was a chance factor.

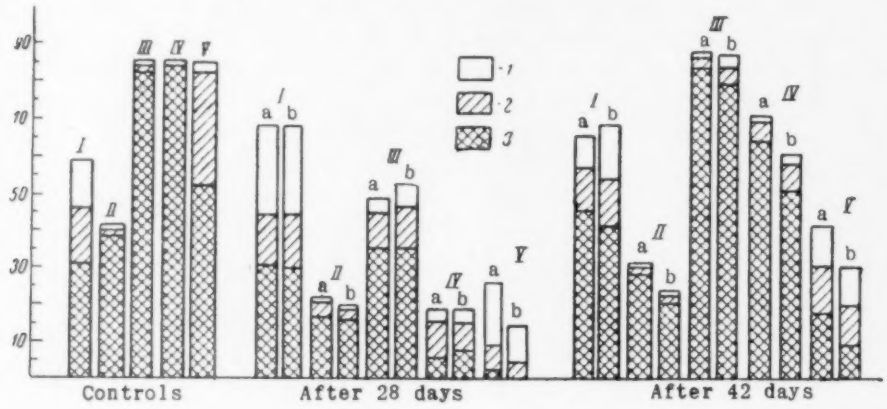


FIG. 1. Change in cell composition of mouse seminiferous tubules after local (a) and whole-body (b) irradiation (400 r). Ordinates, percentage of tubules containing I, spermatogoniae; II, primary-spermatocytes; III, spermatocytes; IV, spermatids; V, sperm. (1) Few cells; (2) Average number; (3) Many cells.

Only the spermatids and sperms had still not been completely restored 42 days after irradiation and were contained in a smaller number of tubules than in the controls. The other types of cells were completely restored. At that time, as also after 28 days, no difference was observed in the histological picture of the testes of the animals exposed to local and whole-body irradiation.

Hence, we failed to detect any difference in the degree of damage to the testes on local and whole-body irradiation. The disturbances observed were due apparently, in the main, to direct damage to the germinal epithelium.

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# FORMATION OF ORGANIC PEROXIDES IN THE LIVER OF RATS IRRADIATED WITH IONIZING RAYS*

V. MAL'TS

Faculty of Fundamental Biology of the Lomonosov State University, Moscow

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VOL. 5 960

SINCE low (in the energy sense) doses of ionizing radiation are capable of producing profound and often irreversible changes leading to death of the irradiated organism, it is quite in order to assume that certain of the initial changes during radiation injury may be intensified.

It is known from the radiobiological literature that the presence of oxygen enhances the processes occurring in radiation injury and that various anti-oxidants are protective substances. All this points to the important rôle of the oxidation reaction in the development of radiation injuries.

It is also known from the literature that, in fats, especially if they contain a large amount of unsaturated fatty acids, auto-oxidizing reactions appear in the presence of oxygen, leading to disintegration of the fats. These reactions occur with formation of lipid peroxides which are intermediate products of the reaction. Anti-oxidants usually present in natural fats and also artificially added to fats are capable of retarding the auto-oxidation reaction.

According to the theory of Semenov, fat oxidation reaction is a branched chain reaction [1]. As Dam and Granados [2] have shown with vitamin E deficiency in the ration of rats, lipid peroxides accumulate in the fats. A number of other workers [3–10] have detected accumulation of fatty peroxides in irradiated rats and mice.

On the basis of the nature of the reaction occurring after irradiation, Tarusov [11–13] has argued that one of the most important reactions occurring in radiation injury is the branched chain oxidation reaction. The intermediate products of the reaction in this case are the lipid peroxides and the end product the free unsaturated fatty acids. These acids accumulating in organs and tissues of the irradiated body are a toxic agent. The substrate of the reaction in the view of Tarusov is found in the cell lipoproteins [11–13].

It was thus of interest to study the kinetics of accumulation of peroxides in the organism after irradiation with different doses.

## MATERIAL AND METHODS

The experiments were carried out on male white rats kept in the usual conditions of an animal house. Irradiation (whole-body, single dose) of the animals was carried

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out from a GUBE-800 unit, with $^{60}Co$ γ-rays in doses of 200, 800 and 10,000 r. At different times after irradiation the rats were killed and the liver extracted in an homogenizer in the presence of *n*-butanol. The homogenate obtained was passed through a G-4 filter. The tissue pulp left behind on the filter was repeatedly mixed with *n*-butanol and again passed through the filter, after which the filtrates were pooled. The peroxides were determined in a mixture consisting of 4 parts butanol extract and 1 part glacial acetic acid. In the presence of peroxides $Fe^{++}$ was oxidized to $Fe^{+++}$, the content of which was determined on a SF-4 spectrophotometer at a wavelength of 302 mμ. On measurement of the $Fe^{+++}$ content a correction was applied for absorption of the extract at this wavelength and also for formation of $Fe^{+++}$ ions in the pure solvent. Binding of the $Fe^{+++}$ ions by the extract was also taken into consideration. The correction for binding of the $Fe^{+++}$ ions by the extract was taken into account in making up the control sample, consisting of the test extract with the addition of a pure solution containing $Fe^{+++}$ ions. Binding of the $Fe^{+++}$ ions by the butanol extracts of the liver of the irradiated rats differed little from the controls. In order to avoid the action of the atmospheric oxygen on the peroxides the whole work was performed in an atmosphere of pure nitrogen. The amount of extracted lipids was determined by weighing them after removal of the butanol by evaporation on a water bath. The peroxides found were estimated in moles per gram extracted lipids.

VOL
5
196

## RESULTS

As the Table and Figure show, the lipid fraction of the liver of the control unirradiated rats contained a low amount of peroxides. Thus, in the control animals the peroxide content was $1{\cdot}6\times10^{-7}$ mol/g with slight individual variations.

After irradiation of rats with a dose of 200 r (not causing death of the animals) the amount of peroxide in the livers started to rise and already on the 1st day after irradiation was $6{\cdot}1\times10^{-7}$ mol/g. The amount of peroxides on the 3rd day after irradiation was also appreciably in excess of the normal level. However, on the 5th day the peroxide content did not differ from the controls and, subsequently, even fell somewhat.

Irradiation of rats with a lethal dose of 800 r led during the first 8 hr after irradiation to a rapid rise in the amount of peroxides in the liver. Then, after a brief reduction in the rate of accumulation of peroxides their content quickly rose, reaching a maximum 3·5 days after irradiation and on the 7th day fell somewhat. The maximum content of peroxides was about $22\times10^{-7}$ mol/g and was detected in the rats at the time they showed the most severe symptoms of radiation sickness which began in the various animals at different times. The different times at which the individual symptoms of radiation injury in the various rats appeared were matched by considerable (as compared with the controls) variations in the content of peroxides in the liver of the irradiated rats on the same day after irradiation (see Table 1).

A dose of 10,000 r, causing death of the rats within 3·5 days, led to an increase in the amount of peroxides in the rat liver, especially in the first 8 hr after irradiation and also on the 3rd day. During the first 8 hr after irradiation there was a continuous

TABLE 1. PEROXIDE CONTENT IN BUTANOL EXTRACT OF LIVER OF IRRADIATED AND UNIRRADIATED RATS

| | Amount of peroxides in lipids (mol/g) $\times 10^1$ | | | | | |
|---|---|---|---|---|---|---|
| | Controls | | | | | |
| Amount of peroxides, $P$ | 1·6 | | | | | |
| $M$ | 0·02 | | | | | |
| Number of animals, $n$ | 19 | | | | | |
| | 200 r (dose rate 62 r/min) | | | | | |
| Time after irradiation, $T$ | 1 hr | 1 day | 3 days | 5 days | 7 days | 11 days |
| Amount of peroxides, $P$ | 2·7 | 6·1 | 4·6 | 0·9 | 1·2 | 1·2 |
| $M$ | — | 0·8 | 0·4 | 0·13 | 0·06 | 0·06 |
| Number of animals, $n$ | 2 | 7 | 8 | 4 | 4 | 4 |
| Statistical significance* | — | + | + | + | + | + |
| | 800 r (dose rate 112 r/min) | | | | | |
| Time after irradiation, $T$ | 2 hr | 7 hr | 1 day | 3 days | 5 days | 7 days |
| Amount of peroxides, P | 3·1 | 4·6 | 6·1 | 22·0 | 21·8 | 15·7 |
| $M$ | 2·2 | 1·4 | 2·9 | 3·5 | 6·2 | 2·0 |
| Number of animals, $n$ | 4 | 7 | 7 | 9 | 10 | 9 |
| Statistical significance* | — | + | + | + | + | + |
| | 10,000 r (dose rate 120 r/min) | | | | | |
| Time after irradiation, $T$ | 1·5 hr | 8 hr | 1 day | 3 days | | |
| Amount of peroxides, $P$ | 16·8 | 20·3 | 7·2 | 19·3 | | |
| $M$ | 1·3 | 9·4 | 0·12 | 6·0 | | |
| Number of animals, $n$ | 4 | 4 | 6 | 9 | | |
| Statistical significance* | + | + | + | + | | |
| | Cysteine + 800 r (dose rate 112 r/min) | | | | | |
| Time after irradiation, $T$ | 1 day | 3 days | 5 days | 10 days | | |
| Amount of peroxides, $P$ | 3·1 | 3·7 | 4·1 | 15·4 | | |
| $M$ | 3·0 | 1·48 | 0·09 | 3·48 | | |
| Number of animals, $n$ | 6 | 10 | 8 | 11 | | |
| Statistical significance* | — | + | + | + | | |

Note. $M = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}}$, where $\bar{x}$ is the arithmetrical mean and $x$ the result of one measurement, $n$ is the number of animals.

* Statistical significance (experimental samples as compared with controls) equal to 99 per cent was computed from formula: $D > 2{\cdot}576\ m_D$ where $D = \bar{x}_1 - \bar{x}_2$ and

$$m_D = \sqrt{\frac{M_1^2}{n} + \frac{M_2^2}{n}}$$

(+) indicates significant results, (—) significance of less than 99 per cent.

increase in the peroxides, the content reaching $20 \times 10^{-7}$ mol/g. Subsequently, the amount of peroxides rapidly fell and already 1 day after irradiation was $7 \times 10^{-7}$ mol/g, which almost coincided with the results obtained on irradiation of rats with doses of 200 and 800 r at this time. Shortly before death of the rats irradiated with a dose of 10,000 r, the amount of peroxides again rose and reached the level of $19{\cdot}3 \times$ $\times 10^{-7}$ mol/g.

On intraperitoneal injection of cysteine (2 ml solution containing 100 mg cysteine in 0·9% NaCl) 5–10 min before irradiation with 800 r, change in the peroxide content differed from that in the normal state (irradiated but no cysteine). However, on the 10th day, the day of the wholesale death of the animals, the peroxide level was almost the same as in the unprotected animals after irradiation with a dose of 800 r.

## DISCUSSION OF RESULTS

As is known from the literature [14] the organs of normal animals always contain low amounts of lipid peroxides. Accumulation of these peroxides is obviously limited by the fact that in the normal body the natural anti-oxidants prevent auto-oxidation of lipids. The group of anti-oxidants includes the vitamins A, E and K, containing lipids in the dissolved form.

As Semenov's experiments [15] show, with oxidation of *n*-decane and *iso*-propylbenzene, a chain reaction only develops if the fall in concentration of the anti-oxidants reaches a definite threshold. Thus, development of a chain reaction in the presence of strong anti-oxidants occurs at low concentrations of these substances and with weak anti-oxidants at higher concentrations.

As our experiments show, in the 1st day after irradiation with doses of 200, 800 and 10,000 r, an almost identical amount of peroxide is found. Retardation in the formation of peroxides in rats irradiated with a dose of 800 r indicates strong inhibition of the chain reactions. Such retardation in the rate of accumulation of peroxides may be due to the fact that there is interaction between the peroxides and anti-oxidants and this slows down the development of the chain process. After destruction of some of the anti-oxidants the chain reaction can now freely develop and the amount of peroxides rises sharply. After irradiation with a dose of 200 r, the chain reaction does not fully develop but fades out. Experiments involving the action of cysteine on the formation of peroxides *in vivo* are in accord with the findings in the literature obtained on models [9]. Injection of cysteine only increases the induction period and does not prevent the development of a chain reaction after the destruction of the anti-oxidants. It is difficult to say why the reaction does not cease in this case. It is possible that on irradiation with such doses, damage is inflicted on the anti-oxidant system so that the chain reaction is no longer regulated. Possible confirmation of this fact may be that on irradiation with high doses and with the use of prophylactic substances an increase in the life span of the animals is observed but complete protection of the body cannot be achieved. The nature of the mechanism of the initial maximum accumulation of peroxides after irradiation with a dose of 10,000 r is not clear. It is possible that in this first period, the peroxides are formed via the direct effect of $\gamma$-rays; subsequently, the peroxides immediately react with the anti-oxidants and after destruction of the latter, a chain oxidation reaction develops.

Another possible explanation for our results is that after irradiation, two different reactions develop, the first being an unbranched chain reaction and the second, a branched one [13]. The curve of peroxide accumulation after a radiation dose of 800 r (see Fig. 1) can be divided into two. The first part corresponds to the rapid increase

in peroxides in the first 8 hr after irradiation which would correspond to the curve for the rise in peroxides at a dose of 200 r. This reaction rapidly subsides if some of the active centres are not restored. Against a background of this first reaction there develops another reaction with a branched chain; the result is that the curve of peroxide accumulation in rats irradiated with a dose of 800 r assumes a sigmoid shape. The curve after irradiation with a dose of 10,000 r might possibly be explained in the same manner. Whether in this process two different reactions or only one occur can only be determined by investigation of the reaction kinetics at different temperatures.

It is significant that in all the animals shortly before death almost equal accumulation of peroxides was detected and therefore, it might be assumed that the peroxides themselves are toxic substances. A series of experiments has been carried out to determine the toxic action of the peroxides [7, 16–24]. This work established that depending on their concentration the peroxides of unsaturated fatty acids, inhibit certain enzymatic

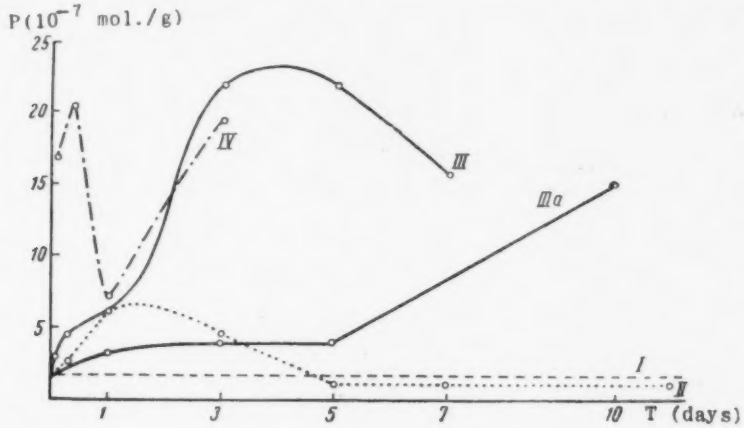


FIG. 1. Time after irradiation ($T$) and amount of peroxides in $10^{-7}$ mol/g extracted lipids ($P$) (I) controls, unirradiated rats; (II) radiation dose 200 r; (III) radiation dose 800 r; (IIIa) radiation dose 800 r after administration of cysteine; (IV) radiation dose 10,000 r.

systems, depolymerize deoxyribonucleic acid, prevent cell division, retard growth of bacteria, fungi and mammals. Injection of peroxides into mice brings about their death. In addition, it has been shown that the peroxides of free unsaturated fatty acids exert a more potent effect than their fatty esters—glyceryl and methyl. There is concentration in the liver of irradiated rats of the free unsaturated fatty acids with potent haemolytic activity [25]. If the peroxides, as these experiments showed, are themselves toxic, then they ought to react with the biosubstrate. However, this would lead to cessation of the chain reaction and the peroxides could not accumulate in such amounts. It is known from the chemistry of fats that at the stage at which the maximum accumulation of peroxides is achieved, disintegration of triglycerides sets in. Therefore, it must be assumed that the substances with toxic action leading to death of the animals

are the peroxides of free unsaturated fatty acids liberated during the chain reactions occurring in the lipid structures of the cells. It is possible that this is accompanied by break-down of a lipoprotein complex. Here we may refer to investigations indicating that in the tissues of irradiated animals subdivision of the lipoprotein complexes occurs [26] and also a reduction in the firmly bound lipids, especially in the cellular organella fraction [27].

## SUMMARY

(1) After whole-body single dose ionizing irradiation of rats accumulation of peroxides of lipids is detected in the liver.

(2) While after a dose of 200 r the amount of peroxides after a brief increase begins to return to normal, at doses of 800 and 10,000 r there is a substantial rise in the peroxide content. After irradiation with a dose of 10,000 r, two peaks are found in the curve of peroxide accumulation.

(3) The reaction has the character of a branched chain reaction.

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VOL. 5 1960

# INVESTIGATION OF THE ELECTRICAL CONDUCTIVITY AND AUTOLYSIS OF THE HEPATIC TISSUE OF IRRADIATED ANIMALS*

YE. V. BURLAKOVA and I. M. PARKHOMENKO

Faculty of Fundamental Biology of the Lomonosov State University, Moscow

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MEASUREMENTS of electrical conductivity have been repeatedly made in investigations on the primary physicochemical processes in tissues on radiation injury [1–4]. The work [4] established continuous changes, for 3–4 hr, in the values of the electrical parameters of the surviving hepatic tissue of control and irradiated animals. With prolonged survival of the liver of white mice an increase in the dispersion of the resistance and in the low-frequency and high-frequency resistances and a reduction in capacitance are observed. In the hepatic tissue of the irradiated mice these changes are greater in absolute terms and occur at a faster rate than in the control animals.

In paper [4] the assumption is made that changes in the electrical parameters of the surviving tissues are paralleled by the processes of autolysis in them. Quite a considerable amount of research has been done on the processes of autolysis in the tissues of irradiated animals and other animals [5–10]. In a number of studies intensification of the processes of tissue autolysis on irradiation has been detected [7, 8]. In others, on the contrary, a decrease in autolysis has been noted [5, 9]. Finally, some investigators have reported both intensification and depression of this process [6]. The discrepancy in the experimental results is possibly to be explained by the different conditions of investigation (for example, the size and intensity of the dose of ionizing radiation, the severity of injury, the physiological state of the organism, methods of measurement of autolysis, etc.). For example, in the work of Kudriashov and Lomsadze [10] both

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an increase and decrease in the processes of autolysis of the hepatic tissue of irradiated rats was obtained depending on the dose and time elapsing after irradiation.

Thus, it may be taken as an established fact, from the published findings, that the character and degree of the autolytic processes *in vitro* are changed in the tissues of irradiated animals. It is highly probable that the changes noted above in the electrical parameters of the surviving tissue of the liver of irradiated animals are associated with change in these processes.

The aim of the present investigation is to establish a correlation between the processes of autolysis of surviving hepatic tissue of irradiated animals and change in its electrical parameters.

## OBJECT AND METHODS

The work was done on the liver of white mice irradiated with $^{60}$Co $\gamma$-rays. A total of four series of experiments was performed. Irradiation was from a GUBE-800 unit, radiation dose 800 and 1000 r, dose rate 500 r/min. The animals after irradiation were kept in the animal house in the same conditions as the controls.

One series of experiments (No. 1) was performed on white rat liver irradiated *in vitro* from a GUT-400–1 $^{60}$Co $\gamma$-unit. In this series the radiation doses were 8000, 25,000, 40,000 and 50,000 r at a dose rate of 350 r/min. The resistance (impedance) was measured on an apparatus specially designed to determine the resistance of live tissues. This apparatus was in the form of a bridge circuit with tuned output and has been described in detail in the work of Burlakova and Kol's [4]. This apparatus makes it possible to measure both the ohmic and the capacitance components of the impedance from an equivalent substitution circuit with parallel switching of capacitance and resistance. The resistance of the tissue was determined at the frequencies 0·2, 2, 10, 50, 100 kc/s and 1 Mc/s. Autolysis was determined by the increase in time of the nitrogen of the free amino acids by the method of Cocking and Jemm [11].

According to this method the nitrogen of the amino acids in the nonprotein centrifugate is determined from the ninhydrin reaction in the presence of a reducing agent (KCN). The amino acids give a colour reaction with ninhydrin the intensity of which was determined on a FEK-52 photoelectrocolorimeter (filter No. 6, 575 m$\mu$). The quantitative content of nitrogen is determined by comparing the results of measurement with a previously plotted calibration curve.

In order to determine the nitrogen an homogenate of hepatic tissue was prepared in distilled water (the homogenate was prepared with cooling in an electric homogenizer). In the second and fourth series of experiments prior to determination, the homogenate was prepared on each occasion from specimens of the liver stored during the experiment in a humid chamber. In the third series the homogenates were prepared at the same time from the liver of the irradiated and control animals and then kept in a thermostat at 37° for 4–7 hr, samples being taken for analysis at definite times. For the resistance measurements, the livers of the control and experimental animals were dissected at the same time in uniform conditions. The mice were killed by decapitation, exsanguinated, the liver extracted, washed with saline, blotted with filter paper and placed

on the tangential platinum electrodes of the humid chamber. The first measurements of resistance were made 15 min after decapitation of the animals, these values being taken as the starting ones. The subsequent measurements were made after 1, 2, 3 and 4 hr. In the series in which the liver was irradiated *in vitro* the liver of the white rats was also placed on the tangential electrodes of the humid chamber in which it was kept throughout the experiment.

## RESULTS

In the first series of experiments a study was made of the change in the value of the low-frequency and high-frequency resistances and the dispersion of the resistance of the rat hepatic tissue irradiated *in vitro*.

VOL.
5
1960

These experiments were performed in order to ascertain whether the electrical parameters of surviving hepatic tissue change on irradiation *in vitro* in a manner similar to that observed for surviving tissue of irradiated animals [4].

The results of this series showed that irradiation of the liver *in vitro* with doses of 8000 and 25,000 r did not produce any changes in the value of the resistance differing from the change in the resistance of the control liver. Only on irradiation with doses of 40,000 and 50,000 r was it possible to detect a marked difference in the character of the change in the electrical parameters of the control and irradiated livers. Changes were noted on measurements at all frequencies. Thus, on measurement at a frequency of 0·2 kc/s towards the end of the experiment, i.e. at 4 hr, the resistance of the irradiated liver was 30 per cent greater than the control, at a frequency of 2 kc/s, 38 per cent greater, at 10 kc/s, 20 per cent, at 50 kc/s, 10 per cent, at 100 kc/s, 8·5 per cent and at 1 Mc/s, 5 per cent (the values are mean values for all the experiments in this series).

Figure 1 shows the resistance of the control and irradiated liver at frequencies of 0·2 and 10 kc/s and 1 Mc/s (experiment No. 1). The abscissa gives the time, the ordinates, the percentage changes in the resistance. As the curve shows, the values of the resistances of the control and irradiated tissue increased with time. Increase in the resistance was greater for the irradiated liver.

The dispersion of the resistance of the irradiated liver also differed from that of the controls. Figure 2 depicts the dispersion of resistance of the liver measured in the 2nd hour of their stay in the humid chamber (experiment No. 1). The abscissa gives the log of the frequency, the ordinates, the values of the resistance in ohms.

It follows from all the experiments in this series that the dispersion of the resistance of the irradiated liver was considerably greater than that for the controls, i.e. the processes occurring in the surviving hepatic tissue irradiated *in vitro* that lead to changes in the electrical parameters are completed within it to a higher degree and at a higher rate than for non-irradiated tissue.

Thus, the pattern of change in the value of the resistance found earlier for the surviving liver of irradiated animals also occurs on irradiation of hepatic tissue *in vitro*. However, these changes, as mentioned above, can be detected only after irradiation with high doses (40,000 and 50,000 r).

Since it has been argued that this increase in resistance occurs in parallel to processes of autolysis [4], special series of experiments were performed (second and third) to

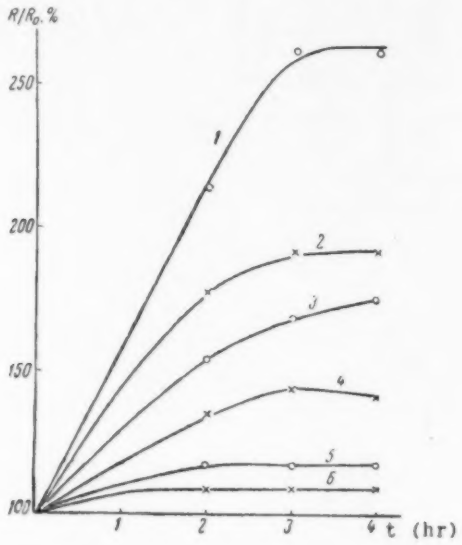


FIG. 1. Change in resistance of liver ($R/R_0$, %) with time (t). (1) Resistance of irradiated liver at frequency 200 c/s; (2) Same, controls; (3) Resistance of irradiated liver at frequency 10 kc/s; (4) Same, controls; (5) Resistance of irradiated liver at frequency of 1 Mc/s; (6) Same, controls.

investigate the processes of autolysis in surviving hepatic tissue and in the fourth series of experiments a parallel investigation was made of the resistance values and the amount

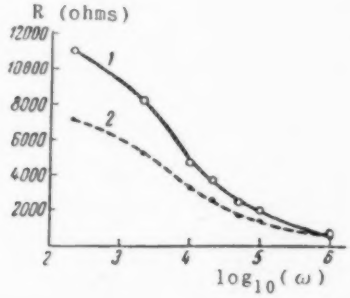


FIG. 2. Dispersion of resistance of hepatic tissue. (1) Dispersion of irradiated liver (40,000 r); (2) Controls.

of free amino acid nitrogen. The results of some of the experiments in the second and third series are given in Table 1.

As Table 1 shows, the amount of the free amino acid nitrogen increased the longer the storage time of the liver or homogenate of hepatic tissue. The increase in nitrogen in the liver of the irradiated animals occurred at a faster rate, i.e. the processes of autolysis in it proceeded with greater intensity than in the controls.

TABLE 1. CHANGES WITH TIME IN THE AMOUNT OF FREE AMINO ACID NITROGEN (IN MG %) IN LIVER TISSUE OF IRRADIATED AND UNIRRADIATED ANIMALS

| Series | Experiment No. | Radiation dose (r) | Increase in amino nitrogen with time (hr) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| II | 1 | 800 | 53·2 | 57·4 | 60·9 | 65·1 | — | — | — | — |
| | | 0 | 48·3 | 50·4 | 53·2 | 53·9 | — | — | — | — |
| | 2 | 800 | 58·8 | 65·8 | 67·2 | 68·6 | 70.0 | — | — | — |
| | | 0 | 52·5 | 54·6 | 55·3 | 57·4 | 58·1 | — | — | — |
| | 3 | 800 | 66·5 | 70·0 | 72·1 | 74·9 | 78·4 | — | — | — |
| | | 0 | 62·3 | 63·7 | 65·1 | 67·2 | 68·6 | — | — | — |
| | 4 | 800 | 62·3 | 66·5 | 69·3 | 72·8 | 74·2 | — | — | — |
| | | 0 | 56·0 | 57·4 | 58·8 | 59·5 | 60·2 | — | — | — |
| | 5 | 800 | 67·9 | 70·7 | 72·1 | 74·2 | 76·3 | — | — | — |
| | | 0 | 57·4 | 58·1 | 58·8 | 60·2 | 61·6 | — | — | — |
| III | 1 | 1000 | 55·9 | — | — | 130·8 | — | 161·4 | — | 203·2 |
| | | 0 | 41·9 | — | — | 100·3 | — | 125·7 | — | 156·3 |
| | 2 | 1000 | 48·2 | 82·6 | — | 141·0 | — | 196·1 | — | — |
| | | 0 | 55·0 | 68·8 | — | 117·0 | — | 161·7 | — | — |
| | 3 | 1000 | 68 | 130 | 184 | 304 | — | — | — | — |
| | | 0 | 54 | 84 | 136 | 204 | — | — | — | — |
| | 4 | 1000 | 81 | — | 150 | — | 249 | — | 345 | — |
| | | 0 | 66 | — | 129 | — | 156 | — | 192 | — |
| | 5 | 1000 | 60 | 99 | 118 | 145 | — | — | — | — |
| | | 0 | 48 | 82 | 102 | 110 | — | — | — | — |

It is interesting to note that autolysis occurred with greater intensity in the tests of the third series where the entire hepatic tissue was homogenized at the start of the experiment. In the second series in which the undamaged liver was kept throughout the experiment in the humid chamber, increase in the amount of nitrogen occured much more slowly. Apparently, the presence of structural living tissue. which can maintained in the first 3–4 hr after removal of the tissue, complicates the processes of autolysis. It follows from the experiments listed in Table 1 that the free amino acid nitrogen content in the hepatic tissue *in vivo* of white mice irradiated with a dose of 800–1000 r apparently also rose somewhat as compared with the controls (in 9 of 10 experiments). However, this difference was small.

In the fourth series of experiments we made a simultaneous investigation of change in the value of resistance and the amount of nitrogen in the liver of the irradiated and control animals. Table 2 shows the difference in the rise in the value of resistance and amino nitrogen in the control liver and that of irradiated animals. The difference

in the increment of resistance is given as a percentage, that of amino nitrogen in mg % (the resistance was measured at a frequency of 2 kc/s).

TABLE 2. CHANGE WITH TIME IN VALUE OF RESISTANCE AND AMOUNT OF AMINO NITROGEN OF THE LIVER TISSUE OF ANIMALS IRRADIATED WITH RADIOACTIVE COBALT

| Experiment No. | Difference in resistance increment in controls and experimental (%) | | | Difference in increment of amino nitrogen in controls and experimental (mg %) | | |
|---|---|---|---|---|---|---|
| | Time in hr | | | | | |
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 7 | 31 | 58 | 2·8 | 5·6 | 7·0 |
| 2 | 39 | 47 | 61 | 2·1 | 4·2 | 5·6 |
| 3 | 16 | 22 | 26 | 2·1 | 4·2 | 4·2 |
| 4 | 52 | 55 | 56 | 3·5 | 5·6 | 5·6 |
| 5 | 14 | 46 | 86 | 2·1 | 3·5 | 4·9 |
| 6 | 29 | 21 | 19 | 0·7 | 2·8 | 2·8 |
| 7 | 56 | 100 | 112 | 3·5 | 4·9 | 5·6 |
| 8 | 7 | 12 | 10 | 0·7 | 2·1 | 3·5 |

The Table shows that increase in resistance was matched by an increase in the amount of amino nitrogen, i.e. intensification of the processes of autolysis in the hepatic tissue of the irradiated animals during the first 2–3 hr after removal of tissue occurred in parallel to an increase in resistance.

In order to establish a relation between these two processes, we computed a correlation coefficient. The correlation coefficient was computed from the formula:

$$r_i = \sqrt{\frac{\Sigma y^2 - \Sigma\Delta^2}{\Sigma y^2}},$$

where $\Sigma\Delta^2$, is the sum of the squares of the deviations of all variants from their group mean values; $\Sigma y^2 = \max \Sigma\Delta^2$, represents the sum of the squares of the deviation of all the variants from their total average $M$. The correlation coefficient was computed for the mean values of all experiments listed in Table 2 and was equal for the 1st hour to 0·88, for the 2nd 0·86 and for the 3rd, 0·81.

The correlation ratio for these values was also computed from the formula recommended for computation for small groups:

$$r_i = \sqrt{r_{i21}^2},$$

$$r_{i21}^2 = \frac{C_{21}}{C_2},$$

where $r_i$ is the correlation coefficient and $C_{21}$ and $C_2$, the dispersions.

The dispersion $C_{21}$ was computed from formula $C_{21} = \Sigma h - H$ and $C_2$ from formula

$$C_2 = \Sigma v^2 - H,$$

where

$$\Sigma h = \Sigma \frac{(\Sigma v_{21})^2}{n} \quad \text{and} \quad H = \frac{(\Sigma v)^2}{n}.$$

In these formulae the letter $v$ denotes the numerical values of the variants and $n$ the number of variants.

The results of the calculations made by both these methods were very similar. Therefore, in discussion of the material we have utilized the results obtained on com putation by one of these methods.

Thus, the value of the correlation coefficient computed for these experiments strictly demonstrates the presence of a definite relation between changes in the processes of autolysis in the first 3–4 hr after removal of the hepatic tissue of irradiated animals and increase in the value of the resistance. The correlation coefficient was not computed for changes in the autolysis process and resistance of the liver in the subsequent hours owing to the fact that the increase in resistance ceased after 3–4 hr survival of the hepatic tissue. At the end of this time, apparently destruction of the living structure, determining the value of electrical parameters of living tissue, set in.

VOL. 5 1960

## SUMMARY

A study has been made of change in the value of low-frequency and high-frequency resistance and dispersion of resistance of the liver of white rats irradiated *in vitro* with radioactive cobalt γ-rays and also the changes in the values of resistance and processes of autolysis of the surviving hepatic tissue of white mice irradiated during life with cobalt γ-rays.

(1) It has been established that on irradiation of the surviving hepatic tissue of white rats with cobalt γ-rays at doses of 40,000 and 50,000 r, an increase occurs in the dispersion of resistance and the value of the low-frequency and high-frequency resistances.

(2) Intensification of autolytic processes in the surviving hepatic tissue of white mice irradiated with cobalt γ-rays at doses of 800–1000 r was detected.

(3) A direct correlation was established between intensification of the autolytic processes of surviving hepatic tissue of irradiated mice and an increase in the value of its low-frequency resistance. The value of the correlation coefficient of these two processes was equal for the 1st hour to 0·88, for the 2nd 0·86 and for the 3rd, 0·81.

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* There is a translation into English of this work.

# DETECTION OF PHYSIOLOGICAL PROTECTION AGAINST IONIZING RADIATIONS IN THE AUTOTETRAPLOIDS OF SEED BUCKWHEAT*

V. V. SAKHAROV, V. V. MANSUROVA, R. N. PLATONOVA and V. K. SHCHERBAKOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
5
196

FOLLOWING the work of Stadler [1] and especially in recent years, a new trend has developed in radiation genetics — the comparative study of the resistance of diploid and polyploid plants to ionizing radiations. Among such studies particular position is occupied by investigations of diploid plants and the autotetraploids derived from them. In this work the utmost accuracy of comparative investigation is called for, since the objects differ only by a multiple of the same original cellular (nuclear) material.

The first indications of raised resistance of the polyploids were obtained on species (wheat, oats, etc.) forming polyploid series, i.e. on natural allopolypoid forms in the formation of which not only polyploidy but also hybridization is involved. The conclusions drawn have been shown to be equally valid for the autopolyploid plants obtained experimentally. In their work [2] Mansurova, Sakharov and Khvostova report raised resistance of the autotetraploids, compared to diploid controls, of the plants of seed buckwheat and millet treated by $\gamma$-rays. Similar findings have been obtained for the autotetraploid rye [3, 4]. The polyploidal forms of lower plants are more resistant to irradiation. The tetraploid forms of yeasts are more resistance than the diploids, the latter in turn being considerably more resistant than the haploids [5, 6]. Moreover, the facts established for plants were found to follow a general biological pattern. In the experiments of Tul'tseva and Astaurov [7] with the diploid forms of the mulberry silkworm and the autopolyploids obtained from them experimentally, it was shown that on passage from a diploid to a triploid and then to a tetraploid state, an appreciable reduction in the damaging effect of irradiation by X-rays was observed. The same also occurs on irradiation of the larvae of the Habrobracon wasp in which nor-

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mally the haploid males and diploid females are compared and also, which is particularly striking, for the haploid and diploid males [8–10].

*Field experiments.* To illustrate the greater resistance of the autopolyploids, we present unpublished results of field experiments (our previous material was based on hothouse experiments on the cultivation of diploid and autotetraploid buckwheat grown from seeds irradiated with γ- and X-rays. The source of γ-radiation was a radioactive isotope of cobalt ($^{60}Co$), the dose rate being 580–600 r/min. The seeds of both buckwheat forms were taken from the previous years crop.

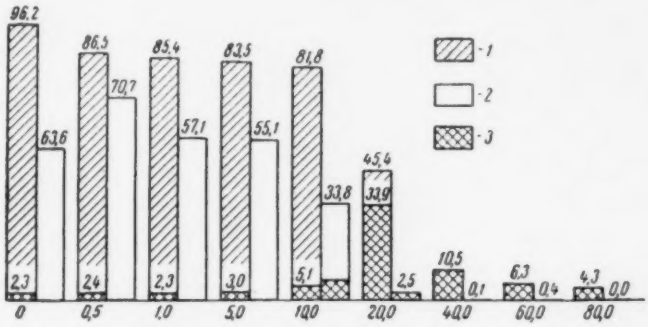


FIG. 1. Percentage germination and survival rate of seedlings of diploid and tetraploid buckwheat for various doses of γ-irradiation. (1) Tetraploid plants; (2) Diploids; (3) Seedlings which died.

Figure 1 graphically depicts the percentage germination of the seeds of the autotetraploids (1) and diploids (2) of buckwheat obtained for the controls and at different doses of γ-radiation (0·5, 1·0, 5·0, 10·0, 20·0, 40·0, 60·0 and 80·0 kr). The Figure shows that for the diploids the critical dose, sharply reducing the percentage of germinated plants, was 10 kr. A sharp drop in germination of the tetraploids was noted for a dose twice as high, 20 kr. It should be emphasized that the critical doses of γ-radiation we established for the same forms of buckwheat grown in hothouse conditions were higher, namely, 20 kr for the diploids and 30 kr for tetraploids. It is natural that the tougher conditions in the field on vegetation of the plants had the effect of reducing the critical doses.

Figure 1 (3) shows the percentage of plants germinating but dying before the 45th day of development when the height of all the plants surviving up to that time was measured. The Figure shows that the raised resistance of the autopolyploids was also manifest in the high survival rates of the germinated plants. At a dose of 20 kr some tetraploid plants survived, at higher doses all the plants died. Very similar phenomena were also observed of the effect of X-rays delivered at doses of 0·5, 1·0, 2·0, 4·0, 8·0, 16·0, 32·0 and 64·0 kr; for the diploids a dose of 8·0 kr was close to the critical and for the autotetraploids a dose twice as high, 16·0 kr. A good criterion of the inhibitory effect of radiations on development is the average height of the control plants and those receiving γ- and X-irradiation. The relevant data for γ-rays are

presented in Fig. 2 and for X-rays in Fig. 3. In both cases the log dose is plotted, the mean height of the plants is expressed in centimetres. In both experimental series a steady fall in the curves was observed, reflecting the inhibitory effect of irradiation

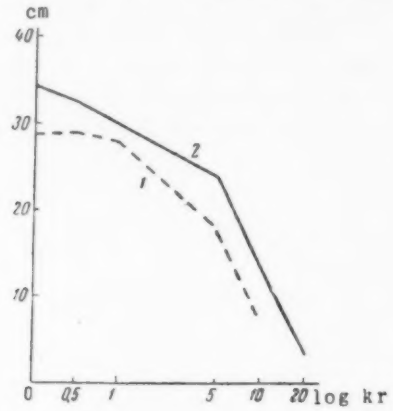


FIG. 2. Effect of various doses of γ-irradiation on depression of growth of diploid and tetraploid buckwheat plants. Abscissa, log doses, kr; ordinates, average height of plants, cm. (1) Diploids; (2) Tetraploids.

on plant growth. The sole exception was a rise in the diploid curve for X-irradiation with doses of 0·5 and 1·0 kr (Fig. 3), this may be attributed to a stimulatory effect of the X-rays. In all cases, except for a questionable one, the radioresistance of the

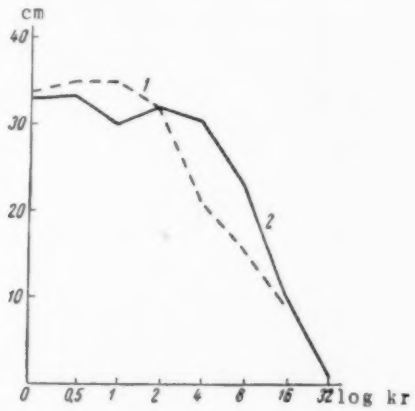


FIG. 3. Effect of various doses of X-irradiation on depression of growth of diploid and tetraploid buckwheat plants. Symbols as in Fig. 2.

autotetraploids was higher than that shown by the diploids. The results of our field experiments are in complete agreement with the findings obtained from cultivation of the plants in hothouse conditions. When the plants were exposed to ionizing radia-

tions the autopolyploids were more resistant than the diploids as judged by all the criteria studied. With an increase in the dose of radiation, germination of their seeds was maintained for a longer time, their seedlings were more viable and, finally, inhibition of growth of the tetraploid plants was manifest only after high doses.

Thus, the phenomenon of raised radioresistance of polyploidal organisms was quite convincingly demonstrated but requires explanation. A fully satisfactory explanation for the raised resistance of polyploids to ionizing radiations was proposed in the work of Stadler [1]. He pointed out that the presence in polyploids of a large number of repeating genomes hinders the manifestation of chromosome disturbances and defects found in each of them. This explanation has received universal recognition. In the paper by Mansurova, Sakharov and Khvostova [2] already mentioned, raised radioresistance of polyploids is fully demonstrated for the autotetraploids of buckwheat and millet; the authors [2] also fully subscribe to the generally recognized and sole point of view regarding the explanation of this resistance. Moreover, in this work a special phenomenon is discussed — spots on the leaves — on the basis of which additional and substantial evidence is brought forward in support of the validity of the genetic explanation of radioresistance of polyploids.

*Cytological investigations.* The genetic explanation for the raised radioresistance of polyploidal plants has received additional proof and confirmation from cytological work. Investigations by foreign workers have shown that because of the presence in the polyploids of a large number of genomes the effect of radiation is to give a correspondingly larger number of chromosome aberrations or cells with aberrations [11–14]. Similarly, in the diploid cells, twice as many chromosome aberrations are formed as in the haploid ones. However, in the presence of a large number of genomes, i.e. a large number of homologous chromosomes, the manifestation of chromosome defects is rendered difficult, both in the irradiated polyploidal forms themselves and in their progeny.

We made a comparative study of the diploids and autotetraploids derived from them in relation to the chromosome changes appearing both in the controls and for various radiation doses. Our extensive cytological investigations, as we shall see below, revealed a new and unexpected pattern.

As in the field experiments, we carried out two series of cytological investigations; one after irradiation with γ-rays and the other with X-rays. The seeds used for the cytological analysis were taken from the same batches which had served for the field experiments.

The germinated seeds were fixed immediately after irradiation, i.e. the times of fixation coincided with those of planting out the seeds in the field. Of course, the doses of γ- and X-irradiation coincided with the doses in the field experiments.

The seeds of both forms of buckwheat were planted at the same time for germination. The rootlet tips were fixed when they reached a length of 5–10 mm. It is precisely in such rootlets that the first pictures of cell division are found in buckwheat.

After 24 hr fixation in a mixture of absolute alcohol and acetic acid in the proportion of 3:1, the rootlets were transferred for prolonged storage in 70 per cent alcohol

or were at once stained with aceto-carmine in which they were kept for not less than 6 hr. After staining temporary squashes were prepared.

On inspection of the preparations under the microscope, only the anaphases of dividing cells were taken into account. Normal anaphases were estimated separately from those with chromosome aberrations. The bridges and fragments observed in the latter were recorded.

The findings of the cytological analysis in the first series of experiments using γ-rays in given in Table 1.

It should be noted that in the controls the percentage of cells with aberrations was quite high for the unirradiated cells stored only 6 months. For the diploid forms, in the cells of which 16 chromosomes were counted, this percentage was 2·2. But precisely the same percentage (2·2) was also noted for the tetraploidal forms with twice as many chromosomes in their cells (32); in the latter case, the percentage of cells with aberrations should have been twice as great if the appearance of chromosome disturbances was equally probable for each of the two genomes of the diploid buckwheat and for each of the four genomes of the tetraploid buckwheat. Bearing this phenomenon in mind, we shall now turn to a consideration of the cytological findings for the irradiated material.

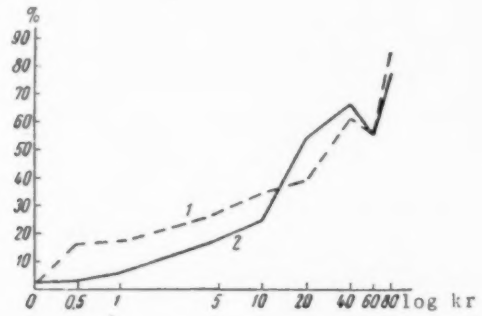


FIG. 4. Percentage of cells with chromsome disturbances due to action of various doses of γ-radiation in diploid and tetraploid buckwheat. (1) Diploids; (2) Tetraploids.

On irradiation of diploid and tetraploid seeds with γ-rays, in both cases there was a fairly regular rise in the percentage number of changed cells (except at a dose of 60 kr) corresponding to the rise in radiation dose (Fig. 4). But again, as in the controls, our findings were unexpected. The tetraploids did not give an increased percentage of changed cells as compared with the diploids, but rather to the contrary, there were less or similar numbers.

The findings of the cytological analysis in the second experimental series, i.e. with X-rays, are given in Table 2 and here, corresponding to a rise in the radiation dose, a quite regular rise in the percentage of changed cells was observed (Fig. 5). As in the preceding series the cytological findings do not allow any conclusion to be drawn

TOL. 5 1960



TABLE 1. CYTOLOGICAL ANALYSIS OF ROOTLETS OF SEEDLINGS DIPLOID AND TETRAPLOID FORMS OF BUCKWHEAT DEVELOPED FROM γ-IRRADIATED SEEDS

| Dose (kr) | Ploidy | No. of anaphases studied | | | | % on conversion to one chromosome | | Ploidy | No. of anaphases studied | | | | % on conversion to one chromosome | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Normal | Changed | % changed | Bridges | Fragments | | Total | Normal | Changed | % changed | Bridges | Fragments |
| Controls | Diploid | 583 | 570 | 13 | 2·2±0·6 | 0·13 | 0·01 | Tetraploid | 873 | 854 | 19 | 2·2±0·5 | 0·06 | 0·02 |
| 0·5 | | 393 | 329 | 64 | 16·3±1·9 | 0·76 | 0·67 | | 257 | 249 | 8 | 3·2±1·1 | 0·15 | 0·09 |
| 1·0 | | 330 | 273 | 57 | 17·3±2·1 | 1·21 | 0·51 | | 293 | 277 | 16 | 5·5±1·3 | 0·17 | 0·05 |
| 5·0 | | 293 | 215 | 78 | 26·6±2·6 | 1·67 | 1·26 | | 434 | 358 | 76 | 17·5±1·8 | 0·59 | 0·10 |
| 10·0 | | 511 | 342 | 169 | 33·1±2·1 | 2·03 | 1·61 | | 807 | 614 | 193 | 23·9±1·5 | 0·73 | 0·64 |
| 20·0 | | 551 | 345 | 206 | 37·4±2·1 | 2·10 | 2·57 | | 1314 | 681 | 633 | 51·9±1·4 | 1·68 | 1·34 |
| 40·0 | | 338 | 138 | 200 | 59·2±2·7 | 4·88 | 4·41 | | 528 | 186 | 342 | 64·1±2·1 | 2·44 | 1·66 |
| 60·0 | | 153 | 72 | 81 | 52·9±4·0 | 4·57 | 5·21 | | 363 | 170 | 193 | 52·9±2·6 | 2·65 | 1·61 |
| 80·0 | | 101 | 19 | 82 | 81·2±3·9 | 8·10 | 7·79 | | 174 | 42 | 132 | 75·2±3·3 | 4·02 | 2·51 |

TABLE 2. CYTOLOGICAL ANALYSIS OF ROOTLETS OF SEEDLINGS OF DIPLOID AND TETRAPLOID FORMS OF BUCKWHEAT DEVELOPED FROM X-IRRADIATED SEEDS

| Dose (kr) | Ploidy | No. of anaphases studied | | | | % on conversion to one chromosome | | Ploidy | No. of anaphases studied | | | | % on conversion to one chromosome | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Normal | Changed | % changed | Bridges | Fragments | | Total | Normal | Changed | % changed | Bridges | Fragments |
| Controls | Diploid | 583 | 570 | 13 | 2·2±0·6 | 0·13 | 0·01 | Tetraploid | 873 | 854 | 19 | 2·2±0·5 | 0·06 | 0·02 |
| 0·5 | | 447 | 399 | 48 | 10·7±1·5 | 0·64 | 0·50 | | 442 | 364 | 78 | 17·6±1·8 | 0·42 | 0·25 |
| 1·0 | | 311 | 278 | 33 | 10·6±1·7 | 0·68 | 0·42 | | 465 | 410 | 55 | 11·8±1·5 | 0·34 | 0·13 |
| 2·0 | | 1098 | 917 | 181 | 16·4±1·1 | 1·38 | 0·21 | | 704 | 574 | 130 | 18·5±1·5 | 0·63 | 0·33 |
| 4·0 | | 740 | 608 | 132 | 17·8±1·4 | 0·94 | 0·75 | | 593 | 392 | 201 | 35·1±1·9 | 1·01 | 0·77 |
| 8·0 | | 733 | 573 | 180 | 23·9±1·6 | 1·03 | 1·67 | | 381 | 222 | 159 | 41·7±2·6 | 1·24 | 1·44 |
| 16·0 | | 289 | 183 | 106 | 36·7±2·8 | 2·72 | 1·75 | | 493 | 290 | 203 | 41·1±2·2 | 1·36 | 0·64 |
| 32·0 | | 662 | 198 | 464 | 70·0±1·8 | 4·80 | 5·66 | | 422 | 215 | 207 | 49·1±2·4 | 2·23 | 1·07 |
| 64·0 | | 360 | 38 | 322 | 89·4±1·5 | 8·01 | 12·30 | | 292 | 66 | 226 | 76·4±2·5 | 3·08 | 3·58 |

on the appearance in the tetraploids of a higher percentage of changed cells than in the diploids under the influence of irradiation. However, at doses of 0·5, 4·0 and 8·0 kr the tetraploids exceeded the diploids, were at the same level for doses of 1·0, 2·0 and 16·0 kr and were less for the highest doses of 32 and 64 kr.

In line with our ideas of the equal probability of formation of aberrations in any of the chromosome sets irrespective of their number in the cell, we would not have expected that the percentage of cells with aberrations in the diploids would have predominated or even a similarity between the percentages of changed cells for both forms of buckwheat. On the contrary, double the percentage of changed cells was to be expected precisely in the tetraploids.

In order to make a detailed comparison of both forms of buckwheat at each dose we made a special calculation of the average percentage of bridges and fragments appearing per chromosome for the diploids with 16 and the tetraploids with 32 chromosomes.

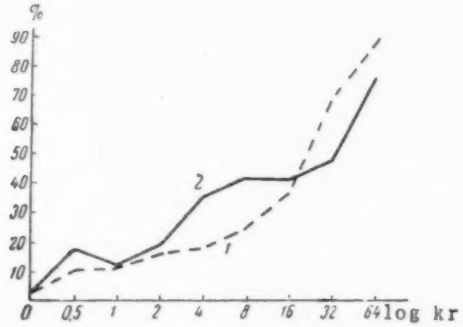


FIG. 5. Percentage of cells with chromosome disturbances due to action of various doses of X-radiation in diploid and tetraploid buckwheat. Symbols as in Fig. 4.

In Fig. 6 where this calculation is made for the series with γ-irradiation, it can be seen that the percentage both of bridges and fragments was greater in the diploid forms for all doses, being double and even higher the percentage for the corresponding changes in the autotetraploids.

This pattern was not so distinctly marked for the series of experiments with X-rays, but here too (Fig. 7), considering at first only the findings for the bridges, it can be seen that the percentages at doses of 0·5, 1·0, 2·0, 16·0, 32·0 and 64·0 kr were about twice as high in the diploids and only for two doses (4·0 and 8·0 kr) were they slightly higher in the tetraploids. In turn, the percentages of fragments at doses of 0·5, 1·0, 16·0, 32·0 and 64·0 kr were in the diploids more than double the corresponding percentages for the tetraploids and only for doses of 2·0 and 4·0 kr did the percentage of fragments in both forms almost coinicide.

Thus, the mean number of injuries produced as a result of radiation per chromosome of the diploid plant was not uniform but was considerably higher than that appearing in the same conditions per chromosome of the tetraploid plant.

As a result of comparative cytological investigations of the diplo- and tetra-forms of buckwheat, the following may be taken as established.

(1) Control, i.e. unirradiated, seeds of both forms stored for 6 months in completely identical conditions gave on germination a quite high, but in the main, completely uniform percentage of cells with aberrations (2·2 per cent).

(2) On germination of the seeds of both forms irradiated with uniform doses of γ- and X-rays, we failed to observe in the tetraploids any regular increase over the diploids in the number of cells with chromosome aberrations. If anything, they were higher in the latter.

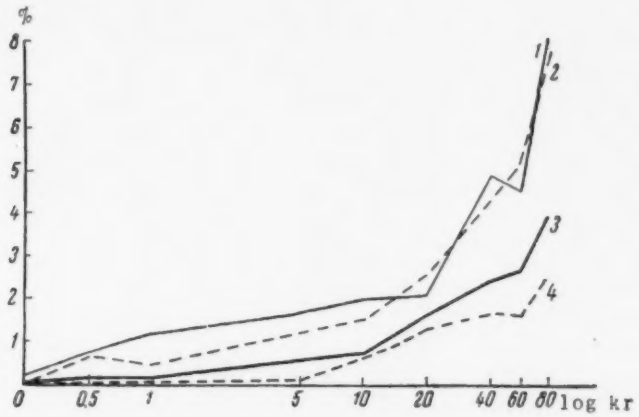


FIG. 6. Average percentage of bridges and fragments per chromosome of diploid and tetraploid buckwheat plants after γ-irradiation. (1) Percentage of bridges in diploids; (2) Percentage of fragments in diploids; (3) Percentage of bridges in tetraploids; (4) Percentage of fragments in tetraploids.

(3) On calculating of the mean percentage of aberrations (bridges and fragments) per chromosome, the tetraploids gave a considerably smaller percentage of disturbances than did the diploids.

These findings are at complete variance with the investigations of the foreign workers noted above. They are also, in consequence, inconsistent with the concept of the equal sensitivity of the chromosome apparatus of haploid, diploid and polyploid cells of the same plant species. However, as early as 1941 in the paper of Sax and Swanson [16] on diploid and tetraploid species of *Tradescantia* it was reported that haploid microspores are something like twice as sensitive as diploids if the chromosome transformations are calculated per chromosome. In their view, the rôle of this factor in the radioresistance of polyploids is more significant than mere change in the number of chromosomes. The work of Sax and Swanson [16] was criticized by Bishop [15] who considered that the differences in sensitivity may be due to differences in the rapidity of development of the haploid and diploid cells. Bishop [15] reaches the usual conclusion that "differences in the sensitivity of the cells and plants as a whole

may be explained as being determined solely by change in the number of chromosomes and not at all by change in the nature of the cell as such".

But the findings of our investigations prompts the suggestion that at the tetraploid level it is precisely the cell itself that changes. Where there could be no doubt of the existence in the polyploids of the phenomenon of genetic protection (larger number of genomes) in our autotetraploids of buckwheat of physiological protection was manifest. The special physiological state of the cell of the autotetraploids of buckwheat limits the possibility of the formation of chromosome aberrations within it.

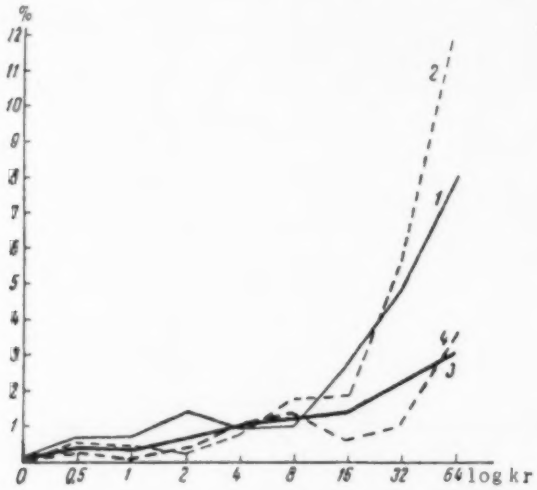


FIG. 7. Average percentage of bridges and fragments formed per chromosome of diploid and tetraploid buckwheat plants after X-irradiation. Symbols as in Fig. 6.

It also determines the high resistance of the polyploid cell itself to ionizing radiations. We can as yet only assume the causes which change the physiological state of the polyploid cell. Possibly the different conditions of moisture content, different levels of enzymatic processes, oxygen lack or other factors hindering the development of chromosome breaks are of significance here.

We repeat that the phenomenon of "physiological protection" does not discount the genetic explanation of the radioresistance of polyploid plants but both must be taken into account.

The phenomenon we established for the autotetraploids of buckwheat seed raises the question of the need for this type of analysis of other polyploids and in the first place experimental autopolyploids.

The findings on the radioresistance of autopolyploid plants can be reasonably compared with our findings on the natural "ageing of seeds".

Comparative study of the ageing seeds of normal and autotetraploid buckwheat seed established the following facts; (a) complete loss of germination of the seeds

of the diploid buckwheat in the 8th year of storage; and (b) germination of the seeds of the autotetraploid form in the 9th year of storage and in a low percentage even in the 10th year.

The same picture was observed for the diploids and our experimental autopolyploids of Tatar buckwheat.

Cytological analysis of the seedlings of the tetraploid buckwheat developed from seeds stored up to the critical period in relation to their germination (9th year of storage) disclosed a considerable percentage of chromosome transformation in the anaphases (27·4 per cent). As for the ordinary buckwheat already in the 3rd year of storage, the percentage of chromosome transformations was more than 5 times as high as in the controls (12·4 as against 2·2 per cent). The findings on the radioresistance of polyploid plants are also of direct practical interest. It is important to take them into account in radiotherapy when normal diploid cells may be selectively killed during the course of such therapy but with retention of the polyploid cells of the tumour. Multiplication of the residual polyploid cells may lead to ineffectiveness in repeat irradiations of the newly-growing tumour [15]. By applying radiation doses critical for diploids cells it is possible to considerably increase the percentage yield of polyploid plants (shoots) among seeds first treated with colchicine. This procedure may considerably simplify work aimed at obtaining experimental polyploids.

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960

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* There is a translation into English of this work.

# RELATION BETWEEN DOSE RATE AND THE RADIOBIOLOGICAL EFFECT IN PLANTS*

A. M. KUZIN, N. M. BEREZINA and O. N. SHLYKOVA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
5
196

THE problem of the effect of the dose rate of radiation is of interest to radiobiologists both from the viewpoint of elucidating the character of the primary processes in the irradiated body and from that of determining the permissible tolerance dose of radiation in work with radiation sources.

In the latter case, as a rule, the result of a large single irradiation is compared with the after-effects produced by the same dose fractionated over a long period. The possibility is assumed of reparative processes in the intervals between the individual irradiations, which according to a number of criteria account for the reduction in the effect of radiation on prolonged fractionated irradiation with a low dose rate as compared with single irradiation with a high dose rate. This problem assumes particular importance at the present time in investigations of the possible danger for living organisms of prolonged exposure to low doses of radiation as a result of global contamination of the earth with the radioactive products of uranium and plutonium fission resulting from nuclear weapon tests [1].

A characteristic feature of these factors is their duration. Theoretically, it may be supposed that prolonged irradiation constantly acting on non-steady systems (such as living organisms) may exert a stronger effect than momentary irradiation for the same total dose. In fact, if a biological effect is induced by a developing short chain reaction in a number of microstructural areas of the specimen being irradiated and the number of these areas at the given moment is strictly limited, although they are constantly formed during metabolism, then the effect from a single dose of radiation will to a considerable degree be limited by the number of these sensitive areas present. For a long-acting source by virtue of the constant formation of sensitive areas in the irradiated object, the overall effect from many transient chain reactions will be considerably greater. This possibility had been referred to earlier [2].

In order to throw light on this situation we carried out experiments with irradiation of dried maize seeds of the Sterling variety at different dose rates.

As criteria of the effects of radiation we took the length of the roots of the coleoptiles and the leaves of the seedlings of the irradiated seeds and also their dry weight.

* Biofizika **5**: No. 5, 566–569, 1960.

We had earlier shown that the growth and development of the seeds are fundamentally affected by the processes occurring in the irradiated endosperm of the grain. We considered that transitory rapidly-breaking chain reactions were probably formed in various tissues of the endosperm, e.g. in the microlipoid structures depending on the metabolic processes. As is known, in resting seeds metabolic processes occur at very low intensity and therefore the time of irradiation has to be considerably increased in order to raise the probability of influencing the different phases of the state of this living system.

The first pilot tests were carried out in 1957. Air-dried seeds of maize of the Sterling variety were placed 54 cm away from a $^{60}Co$ source with an activity of 1 curie. Irradiation was carried out for 96 days at a dose rate of 4·6 r/hr. The seeds received in all 11,000 r. The second batch of seeds of the same sample was subjected to irradiation for the same dose of 11,000 r but at a dose rate of 600 r/min. Then the seeds irradiated with the different dose rates simultaneously with the unirradiated seeds of the same batch were spread on Petri dishes at the rate of 25 per dish. The experiments were run in five sets. Two experiments were carried out. The roots were measured on the 5th, 7th, 12th and 14th day. The average data for 250 measurements are given in Fig. 1.

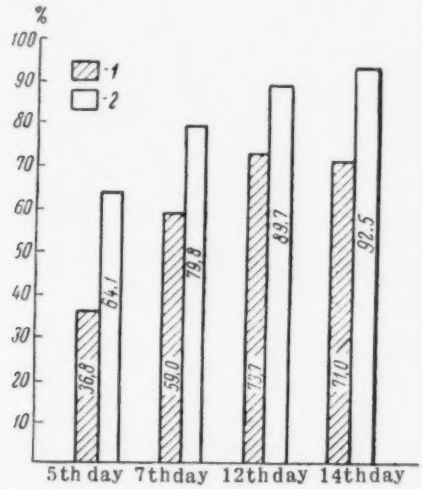


FIG. 1. Length of maize seedling roots (% of controls) on prolonged and short irradiation with a dose of 11,000 r at various times after sowing (1957 experiment). (1) Prolonged irradiation; (2) Short irradiation.

As the Figure shows, prolonged irradiation exerted a distinctly greater depressant effect than short irradiation, especially in the early stages of germination.

In 1959, an experiment was carried out in the following conditions. The air-dried seeds of maize of the Sterling variety were placed in polymethacrylate containers so designed as to completely protect the seeds from rainfall and the sun's radiation. The apertures in the walls of the containers allowed free access of the surrounding

air to the seeds. The containers, attached to special tripods, were set in the γ-field at a height of 80 cm from the surface and a distance of 5·2 m from the radiation source of $^{60}Co$ with an activity of 210 g/equiv. radium. The dose rate at a distance of 5·2 m from the source was 6·4 r/hr. The experiment in the γ-field lasted from 30 May to 12 October. During this time the seeds were irradiated for 2126 hr, for the remainder of the time the cobalt source was shielded in carrying out the field work. The total time during which the cobalt was shielded per month was from 30·4 to 35·8 per cent of the total time. The total radiation dose was 13,600 r. At the same time as the seeds were placed in the γ-field some seeds of the same batch, intended for short term irradiation and controls, were kept in the same meterological conditions, i.e. stored outside the laboratory. The day before completion of the experiments in the γ-field,

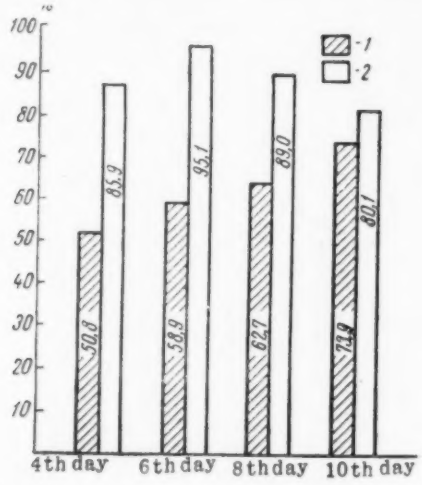


FIG. 2. Length of roots of maize seedlings (% of controls) on prolonged and short irradiation with dose of 13,600 r at various times after sowing (mean data of 2 experiments in 1959). (1) Prolonged irradiation; (2) Short irradiation.

some of these seeds were briefly irradiated with a dose of 13,600 r. Irradiation was carried out with $^{60}Co$ γ-rays at a dose rate of 500 r/min and the accuracy of dosimetry was 5 per cent. The seeds were then spread 25 to a dish in five sets in Petri dishes. The measurements of the roots, coleoptiles and leaves were made on the 2nd, 4th, 6th, 8th and 10th day of vegetating. In order to follow the growth and development of the seeds for a longer period — 20 days — some seeds were grown on polymethacrylate meshes in glasses. At the end of 20 days, the length of the roots and leaves of these seedlings was measured and also their dry weight determined. On the 20th day, it was found that in the plants irradiated for a short time, 28·7 per cent had 3–4 additional roots, while the plants irradiated for a long time in the γ-field had not such a high number — only 2 additional roots in 5·5 per cent; this again indicates that

they lagged behind the seedlings irradiated only for a short time in growth and development. The investigation described here was carried out on two occasions with concordant results. Figure 2 gives the results of measurements of the length of the roots for prolonged and short irradiation of the seeds with a dose of 13,600 r (average of 250 measurements in each of two experiments). As the results show, in all cases prolonged irradiation exerted a much more powerful depressant effect on growth and development of the seedlings than did brief irradiation with the same dose.

Figure 3 presents the findings on the dry weight of the roots and leaves on the 20th day of vegetation, again much greater depression of root and leaf growth was observed with prolonged irradiation. On average, the effect of prolonged irradiation at a dose of 13,600 r was about 20 per cent greater than for short irradiation.

VOL. 5 1960

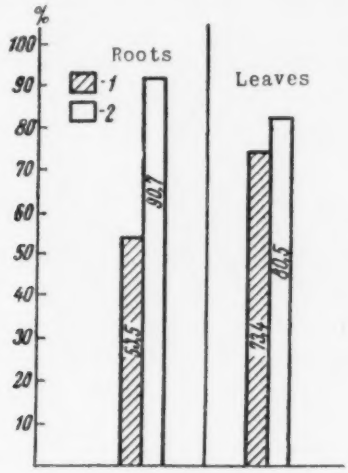


FIG. 3. Dry weight of roots and leaves of maize seedlings (% of controls) on prolonged and short irradiation on 20th day of vegetation. (1) Prolonged irradiation; (2) Short irradiation.

Depression of the development of the root system, in comparisons of the dry weight, increased on the 20th day up to 50 per cent. This was due to the fact that on prolonged irradiation development of additional roots was at this time depressed. The results show that prolonged irradiation impairs the vegetative functions in irradiated seeds to a greater extent than does brief irradiation with the same dose.

The particular features of prolonged as compared with brief irradiation which we have demonstrated call for great caution in assessing the safety of high radioactive levels of the environment when judged in terms of the overall dose for a long period of time without taking into account the intensification of the effect by reason of the non-steady state of the irradiated living organisms. It should be emphasized that we detected differences on extending the irradiation time 3 months. It may be expected

that this is still not the maximum period and that the intensification will be even greater for longer intervals of time. Investigations in this direction are now under way.

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# INTENSIFICATION OF EFFECT OF X-IRRADIATION ON SEEDS DURING STORAGE*

I. M. VASIL'EV, B. G. ZHUKOV and T. S. SPASSKAIA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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As far back as 1910, Guilleminot [1] showed that on storage for a year of seeds irradiated by X-rays or radium, their germination is reduced to a greater extent than for unirradiated seeds. The effect of irradiation, as it were, combines with the natural loss of germinating ability of seeds on storage. Later, Tascher [2] observed the same effect of irradiation. He stored barley seeds irradiated with X-rays at room temperature for 12 weeks and found that the percentage of germinating seeds invariably diminished with increase in the time of their storage. The same results were obtained by Stadler [3] and Ehrenberg [4] and, in recent times, in the experiments of Adams and Nilan [5] and of Vakar, Kaloshina and others [6]. Adams and Nilan irradiated resting barley seeds with 7500 r X-rays and then stored them together with unirradiated seeds for 2, 4, 6 and 48 months. Storage of the irradiated seeds in air led to an increase in chromosome aberrations, reduction in the energy of germination, inhibition of growth and reduction in the number of viable plants by the time of maturation. Storage of the seeds in an atmosphere of oxygen increased the damage; storage in nitrogen reduced it. In the experiments of Vakar, Kaloshina *et al.*, storage of irradiated wheat and maize seeds increased the number of non-germinating seeds.

There are also indications of a lessening of the effect of irradiation during storage of irradiated seeds. Such findings were obtained in the investigations of Salser [7] in which irradiated wheat and barley seeds stored for 4 months resulted in plants with less damage then did freshly irradiazed seeds. (The same results were also obtained in our investigations when for the experiments "old" seeds stored for several years after harvesting were used).

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VOL
5
196

Let us consider the results of our investigations. The seeds of winter wheat 599 were irradiated in the air-dried state with X-rays at doses of 1000, 5000, 10,000, 25,000, 50,000 and 100,000 r and placed together with unirradiated seeds for storage in a cupboard at room temperature. After 6 months, previously unirradiated seeds were irradiated and immediately after this all the batches of seeds — unirradiated, irradiated

FIG. 1. Winter wheat 599. In each photograph from left to right: plants from unirradiated seeds, from freshly irradiated seeds and from seeds stored after irradiation for 6 months. Radiation dose: (1) 10,000 r; (2) 25,000 r.

6 months previously and the freshly irradiated — were soaked in tap water and grown at room temperature; from the 2-day old seedlings the better ones were selected, spread on polymethacrylate meshes and kept for 6 days in Knopf's nutrient solution in a luminostat at a constant temperature of $23 \pm 2^{\circ}$ under DS-30 lamps.

The experimental results showed that irradiation with a dose of 1000 r had no effect on the plants grown from the freshly irradiated seeds and made little difference to plants grown from seeds stored after irradiation. Irradiation with a dose of 5000 r had the effect of slightly suppressing the growth of the plants from the freshly irradiated seeds while the depressant effect on plant growth in the case of seeds stored after irradiation was even greater. A dose of 10,000 r gave considerable inhibition of plant growth from freshly irradiated seeds and even greater inhibition for those stored after irradiation. A dose of 25,000 r sharply inhibited growth of plants from freshly irradiated seeds. Nevertheless, the plants did continue to grow. Plant growth in the case of seeds stored after irradiation was completely inhibited. The seeds germinated, as is usually the case, even after irradiation with very high doses (running into millions of röentgens) but their subsequent development was arrested. There was no further growth associated with accumulation of protoplasm and cell division, but only elongation of the cells of the embryonic leaves and roots usual in germination. A dose of 50,000 r completely inhibited growth in the irradiazed seeds of both batches. They only germinated, as did the seeds stored in the irradiated state after 25,000 r. The same result was given by a dose of 100,000 r. After a certain time the experiment was repeated. The freshly irradiated seeds and those stored for 2·5 months after irradiation were compared with similar results. The effect of storage was not quite so markedly expressed.

In the third and similar experiment, freshly irradiated seeds and those stored for 1·5 months after irradiation were compared. The effect of storage was even less marked. It was negligible in the plants grown from seeds irradiated with 1000, 5000 and 10,000 r but was well to the fore in those grown from seeds irradiated with doses of 25,000 r. Seeds irradiated with doses of 50,000 and 100,000 r in both cases gave a uniform effect since growth of the seedlings was completely inhibited.

It follows from the experiments described that storage of irradiated wheat seeds intensified the effect of radiation in relation to inhibition of plant growth. This effect increased with the time of storage of the irradiated seeds. It was greatest for seeds stored 6 months, somewhat less on storage for 2·5 months and patently less for those stored 1·5 months.

Assessment of these results, which agree with those of many other similar investigations, is based on the following factors. On natural storage of seeds their viability diminishes with passage of time with a corresponding reduction in the energy of germination and subsequent growth. Ionizing radiations act on the seeds in a similar manner. They, as it were, speed up the ageing of the seeds. On storage of irradiated seeds, natural ageing is intensified by the effect of irradiation so that more marked ageing results.

The idea of accelerating ageing of the organism after irradiation is not a new one. Support for idea was provided by Nadson [8] and Nemenov [9–10]. The findings described above give additional support.

VOL
5
196

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VOL.
5
1960

# EFFECT OF ULTRASONIC RADIATION ON RIBONUCLEASE *

I. YE. EL'PINER and O. M. ZORINA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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ENZYMES dissolved in an aqueous medium in the field of ultrasonic waves are subjected as a rule to a process of inactivation. Loss of activity is observed as a result of ultrasonic irradiation of pepsin and trypsin [1, 2], invertase [3], cholinesterase, deoxyribonuclease [4], hyaluronidase [5] and others. Inactivation of most of these enzymes is detected only when irradiation is carried out in the presence of oxygen. Ultrasonic irradiation of an enzyme does not lead to its inactivation if the test solution has been first saturated with other gases; hydrogen, nitrogen or helium. An exception is argon, which is able in certain cases to intensify the oxidative processes due to ultrasonic waves [6]. As our previous investigations [7] showed, proteolytic enzymes in a field of ultrasonic waves are inactivated not only in the presence of oxygen but in the presence of argon. Inactivation of the enzymes in this case is accompanied by a reduction in the absorption band maximum in the ultra-violet region of the spectrum and diminution in the molecular weight of the protein under study. In the presence of hydrogen the enzymatic activity of pepsin and trypsin is not diminished nor does any change occur in the corresponding spectrophotometric curves. In these cases only the molecular weight of the irradiated proteolytic enzyme increases.

These investigations point to the possibility of studying along these lines the existing correlation between the enzymatic properties of a protein and the special features of its structure. The experimental material set out in the present paper also indicates

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this possibility. The test object in our investigation was the enzyme ribonuclease, the structure of which has been more fully studied than the structure of the proteolytic enzymes listed above. The ribonuclease molecule is in the form of a single polypeptide chain consisting of 124 amino acid residues. The individual molecular units are cross-linked through four disulphide bonds. However, data on the structure of the active centre of ribonuclease are very conflicting. There are indications that the activity of the enzyme is due to the histidine part of the protein. According to a number of investigators [8–10] on photo-oxidation of two of the four histidine residues of the enzyme molecule or on blocking of the latter by iodoacetate, enzymatic activity disappears. A special rôle in the activity of ribonuclease has been assigned to the disulphide bonds since loss of activity of this enzyme is observed on oxidative or reduction rupture of these bonds [11, 12]. Latterly, there have appeared papers [13] indicating that with rupture of the disulphide bonds the secondary structure of the protein molecule is disturbed with which apparently the activity of the enzymes is partially associated.

In the view of Anfinsen [14] and Bigelow [15] the activity of ribonuclease diminishes on rupture of the hydrogen bonds between the tyrosine residues adjacent to the C-terminal ring which is accompanied here by disturbance in the secondary structure of the proteins.

As will be shown, some ideas of the link between the structure and the function of ribonuclease may be gained by analysing the changes observed on irradiation of this enzyme with ultrasonic waves.

A 0·01% aqueous solution of ribonuclease was subjected to ultrasonic irradiation. The frequency of the ultrasonic generator was 670 kc/s, intensity 8 W/cm$^2$. The ribonuclease activity was determined by the method of Anfinsen [14]. The enzyme-substrate ratio (ribonuclease-ribonucleic acid) was 1:600; the activity of the enzyme was determined at pH 6·2 (0·1 N acetate buffer). The precipitant of the areactive substrate was 0·25% solution of uracil nitrate in 10% $HClO_4$.

It was found that irradiation of ribonuclease in the presence of air for 30 min did not lead to reduction in the activity of the enzyme. It was established spectrophotometrically that a result of an increase in the duration of treatment with ultrasonic waves was a reduction in the number of cyclic amino acid residues. This was expressed in a reduction, for 30-min irradiation even disappearance, of the absorption maximum specific for protein in the u.v. at $\lambda = 2800$ Å (Fig. 1). The molecular weight of the enzyme irradiated in the presence of oxygen also showed no change (measurements were made on Wilhelm scales, we wish to express our thanks to G. A. Deborin for valuable advice and assistance in carrying out the measurements of the molecular weight).

Nor was there any fall in the activity of the enzyme when irradiation was carried out in the presence of hydrogen. However, in these conditions no reduction in the protein absorption maximum in the ultra-violet region of the spectrum was observed. As Table 1 shows, the molecular weight of ribonuclease irradiated in the presence of hydrogen for 30 min increased by 40–45 per cent as compared with that of the original protein. Thus, unlike the proteolytic enzymes, the activity of ribonuclease did not fall despite the fact that in the presence of oxygen a diminution in the optical density

of the enzyme under test was observed. This means that the activity of ribonuclease is apparently not associated with the cyclic amino acid residues in the molecule of this protein.

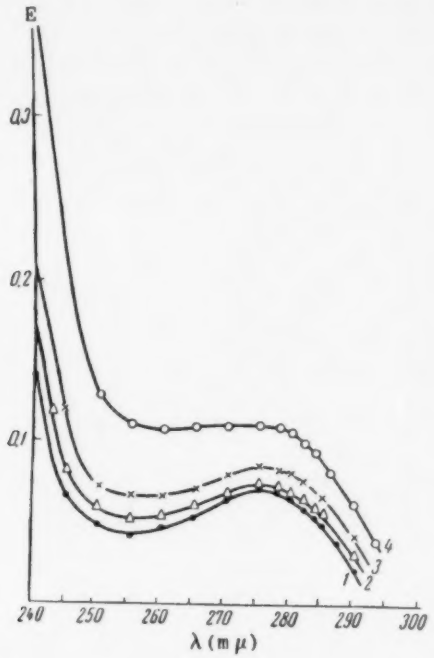


FIG. 1. Optical density (E) of aqueous solutions of ribonuclease as a function of duration of irradiation (concentration of enzyme 0·01 per cent). (1) Unirradiated enzyme; (2) Duration of irradiation 2 min; (3) 5 min; (4) 30 min.

Another interesting fact was that, as distinct from the proteolytic enzymes, the molecular weight of ribonuclease irradiated in the presence of oxygen did not fall. We would recall that the molecular weight does not fall if irradiation is carried out of macromolecules with a molecular weight less than 25,000–20,000. The molecular weight of the ribonuclease used by us was 17,000.

TABLE 1. VARIATION OF MOLECULAR WEIGHT OF RIBONUCLEASE WITH DURATION OF IRRADIATION IN PRESENCE OF HYDROGEN

| Enzyme | Duration of irradiation (min) | Molecular weight of enzyme | Change in molecular weight (%) |
|---|---|---|---|
| Ribonuclease | 0 | 17,300 | 0 |
| ,, | 30 | 24,000 | 45 |
| ,, | 60 | 19,000 | 15 |
| ,, | 120 | 17,000 | 0 |

This does not mean apparently, that this enzyme, with a comparatively low molecular weight, did not undergo chemical changes in a field of ultrasonic waves.

There are grounds for believing that the changes in structure of the protein molecule possible in the presence of oxygen (tearing away of the side or end groups), but not reflected in the activity of the enzyme and in its molecular weight, did not lead to further chemical changes of the protein. It is possible that oxygen, in this case, inactivated the protein radicals formed as a result of ultrasonic waves.

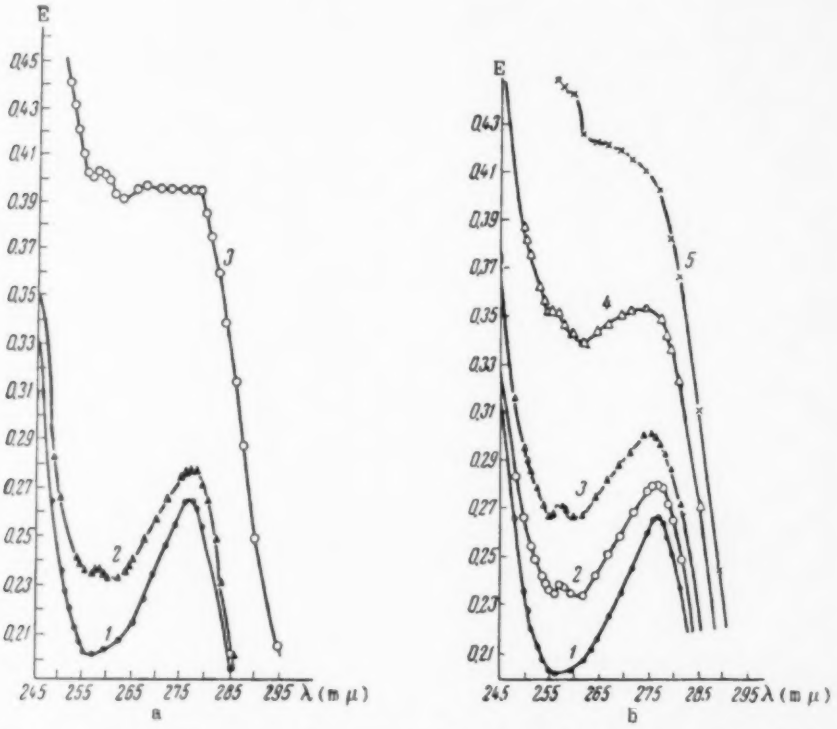


FIG. 2. (a) Optical density E of serum albumin on irradiation in alkaline medium (pH 9·18) as a function of the nature of gas present (concentration of protein 0·04 per cent). (1) Unirradiated protein; (2) Irradiation in presence of hydrogen; (3) In presence of air. (b) Optical density E of serum albumin on irradiation in alkaline medium (pH 9·18) in presence of hydrogen as a function of duration of irradiation (protein concentrations 0·04 per cent). (1) Unirradiated protein; (2) Duration of irradiation 30 min; (3) 60 min; 4, 120 min; (5) 240 min.

Activation of the macromolecule in this respect occurred if the radiation of the aqueous solution was carried out in the presence of hydrogen. This explains why in the presence of hydrogen the activated macromolecules formed, or possibly, their fragments interact with each other. As a result of this, the mean molecular weight of the protein studied increased by 1·5 and more times; for a longer duration of irradiation the process of splitting off of the macromolecules began to prevail (Table 1).

It was found (Fig. 2 (a) and 2 (b)) that on irradiation of ribonuclease or serum albumin there occurs not only an increase in the molecular weight of the protein irradiated in the presence of hydrogen, but in the solution there also appear low molecular weight particles of the size of small peptides or amino acids, the number of which increased with the duration of irradiation. This was also observed on irradiation in the presence of air; and in this case, it was more distinctly expressed.

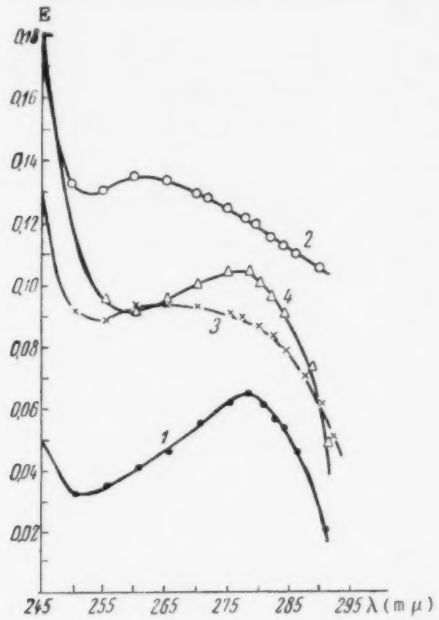


FIG. 3. Variation of optical density E of ribonuclease, on irradiation in air, with pH of medium in which irradiation carried out (concentration of enzyme 0·02 per cent). (1) unirradiated solution; (2) irradiation in buffer at pH 9·18; (3) pH 7; (4) pH 4·65.

As was shown, the character of the spectrophotometric curves varied considerably with the pH of the phosphate buffer in which the protein solution was irradiated in the presence of air. As Fig. 3 shows, with an increase in the pH the specific absorption band typical of proteins was considerably extended and the maximum of this band became flatter.

Analysis of these curves led to the conclusion that in this case it is most likely that there exist two absorption bands with two maxima (at 2800 Å and at 2600–2500 Å, partially superimposing on each other). The second absorption band at the higher frequencies (2500–2600 Å) was apparently due to the appearance of amino acid or small peptides. As was previously shown by El'piner and Sokol'skaia [16], the aliphatic amino acids become able to adsorb ultra-violet energy in the 2500–2600 Å region if they are subjected to ultrasonic irradiation in alkaline medium or if spectrophotometric investigation is carried out in alkaline medium (phenomenon of cyclicization).

If, according to the assumption put forward above, in the irradiated solution of ribonuclease, low molecular weight protein fragments appear, these particles may be removed by dialysis. In fact, dialysis of the irradiated enzyme against the buffer used resulted in the dialysate giving a spectrophotometric curve characteristic only of the protein solutions (Fig. 4). In other words, during dialysis, the low molecular particles responsible for the appearance in alkaline medium of the second peak at 2500 or 2600 Å and torn off from the protein, were removed from the solution.

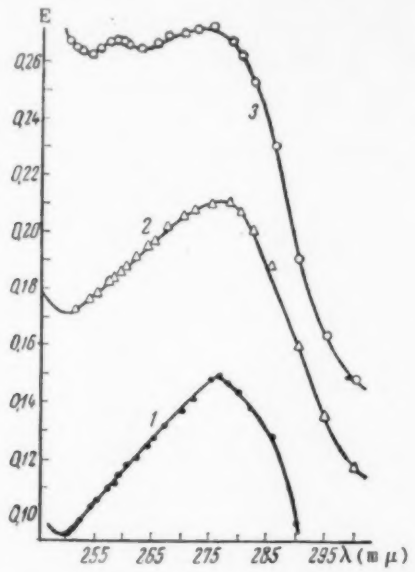


Fig. 4. Optical density E of ribonuclease irradiated in alkaline medium (pH 8·05) before and after dialysis. (1) Unirradiated solution; (2) Irradiated solution after dialysis; (3) Same solution before dialysis.

It is important to note that the activity of the enzyme irradiated in the presence of hydrogen both before dialysis and after it, remained unchanged.

The findings reported show that ribonuclease in a field of ultrasonic waves, on the one hand, retains its enzymatic activity and, on the other, undergoes, with comparative ease, chemical changes accompanied even by tearing away of low weight molecular particles from the enzyme molecules. This corresponds to certain findings in the literature. Thus, for example, according to Kalnitsky and Rogers [17], splitting off from the ribonuclease (with carboxypeptidase) of a number of amino acids — even up to 15–20 per cent of their total number — does not lead to destruction of enzymatic activity. It is of exceptional interest that in the non-enzymatic, purely physical (or physicochemical) splitting off from the enzyme of the low molecular weight particles, its activity is not lost. In conclusion, it is important to note that irradiation of ribonuclease in the presence of argon was accompanied by a certain reduction in its enzy-

matic activity which indicates the possible rôle of the OH-radicals in the inactivation of the enzyme, confirming the results of Collinson and Dainton [18]. This will be the subject of our further research.

## SUMMARY

(1) Ribonuclease irradiated with an intensity of ultrasound (10 W/cm$^2$) did not lose its enzymatic activity in our experiments. Loss of activity was not observed on irradiation of the enzyme either in the presence of hydrogen or oxygen.

(2) However, irradiation of ribonuclease was accompanied by considerable chemical transformations of the protein molecule, expressed in splitting off from the protein molecule of low weight molecular peptide or amino acid residues. In addition, in the ribonuclease solutions irradiated in the presence of oxygen, lowering of the absorption band maximum in the ultra-violet spectrum was observed.

(3) As for ribonuclease irradiated in the presence of hydrogen, in these conditions an increase in the molecular weight of the enzyme was noted (on average by 40–50 per cent).

(4) Analysis of the phenomena described suggests that the disintegration of the cyclic amino acid residues of the ribonuclease molecule does not lead to reduction in its enzymatic activity as in the case of proteolytic enzymes. Nor is the splitting off from the molecule of low-molecular weight peptide or amino acid fragments reflected in the enzymatic activity of the ribonuclease.

VOL.
5
1960

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* There is a translation into English of this work.

# EFFECT OF IONIZED SIDE GROUPS ON THE MAGNETIC PROPERTIES OF RIBONUCLEIC ACID*

L. V. VOZYSHAYEVA and L. A. BLIUMENFEL'D

Institute of Chemical Physics, U.S.S.R. Academy of Sciences, Moscow

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As a result of previous investigations of the electron paramagnetic resonance spectra (e.p.r.) of deoxyribonucleic and ribonucleic acid (DNA and RNA) and the RNA protein complexes [1], the appearance of very broad e.p.r. lines with an integral intensity characterized by strong asymmetry and sometimes by absorption in the zero magnetic field was demonstrated.

Similar effects have been detected for certain types of synthetic polymers [2].

Analysis of the structures of these compounds showed that the appearance of anomalous magnetic properties is possibly associated with the presence of orderly-arranged polar groupings in the polymer chain [3].

It is known that the molecules of nucleic acids possess a high number of ionizible acidic and basic groups, the number and sign of which depend on the pH of the medium. For acid pH values the acidic groups (phosphoric acid residues) are in the non-ionized form and the molecules as a whole are positively charged, chiefly owing to ionization of the amino and imino groups:

$$-NH_2 + H^+ \rightleftharpoons NH_3^+$$

For alkaline pH values the molecule bears a negative total charge owing to ionization of the acid groups:

$$-HPO_3- \rightleftharpoons PO_3^- + H^+$$

At pH values corresponding to the isoelectric point the molecule of the polymer bears a total zero charge, the number of charged groups is minimal and the number of negatively-charged equal to the number of positively-charged groups. It was therefore of interest to verify the relationship between the magnetic properties of the RNA preparation and the pH of the medium at which it was isolated.

For this purpose we investigated the e.p.r. spectra of dry RNA preparations obtained at different pH values of the medium. The RNA was obtained by the method of Mallte and Lamanna [4]; apart from the procedures indicated by the authors we employed additional purification, consisting in dissolving the preparation obtained in 400 ml

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water with subsequent spinning down of the insoluble residue. The supernatant was dialysed against water for 20 hr (water was changed 4 times) and dried by lyophilization for 18 hr. Chemical analysis showed the nitrogen content of the RNA preparation, was 13·93 per cent and the phosphorus 8·6 per cent; N/P = 1·62. The kinematic viscosity of a 0·3 per cent solution of RNA was 1·240 cs.

The initial solution was prepared by dissolving a sample of dried RNA preparation in 0·2 N NaCl (1 mg RNA per ml solution). By adding a suitable amount of 0·1 N HCl or 0·1 N KOH the pH of the samples was adjusted to the following values:

| | |
|---|---|
| 2·0–2·2 | 5·8– 6·0 |
| 3·8–4·0 | 8·0– 8·2 |
| 5·0–5·2 | 10·0–10·2 |

VOL. 5 1960

The pH of the solution was measured in a LP-5 type potentiometer with a glass electrode. The samples thus prepared were dried by lyophilization. Accurately weighed samples of the dried preparations were placed into glass ampoules and their e.p.r. spectra measured.

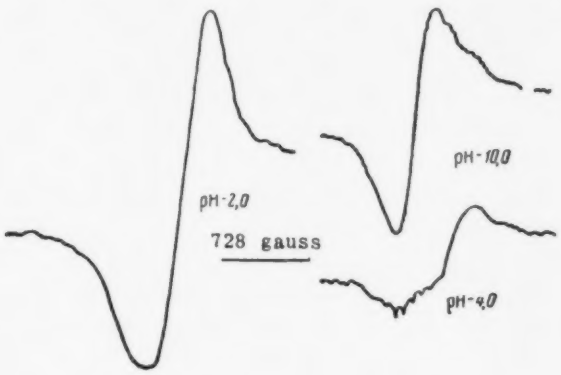


FIG. 1. E.p.r. spectra of RNA preparations at pH 2·0; 4·0 and 10·0.

The e.p.r. spectra were measured on a EPR-2 type spectrometer to the IKhF design of Semenov with high frequency modulation [5]. The sensitivity of the apparatus was $5 \times 10^{12}$ paramagnetic particles in a sample of 1:1-diphenyl-picryl hydrazine.

Figure 1 shows the spectra of the dried preparations made up at pH = 2·0, 4·0 and 10·0.

As the Figures show, RNA gives broad asymmetrical e.p.r. lines with a width between points of maximal slope of 400–600 gauss with a g-factor of 2·3. The intensity of the signal was minimal for the preparations obtained at pH = 4–6 and sharply rose on both sides of this value.

Figure 2 shows the curve relating the relative intensity of the signal to the pH.

As follows from these observations, for the pure RNA preparations the appearance of orderly-arranged positively or negatively-charged groups in the chain was accom-

panied by a rise in the value of the effect whereas at the isoelectric point (approximately at pH = 4·3) the magnitude of the effect fell considerably.

In order to confirm our findings the isoelectric point of our RNA preparation was determined by titrating it with 0·1 N KOH and 0·1 N HCl and also according to its solubility.

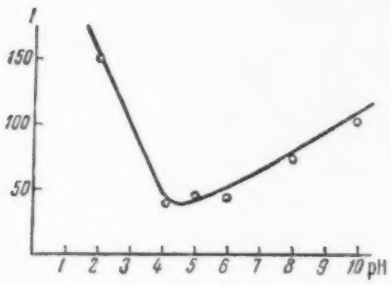


FIG. 2. Curve relating relative intensity I of signal to pH.

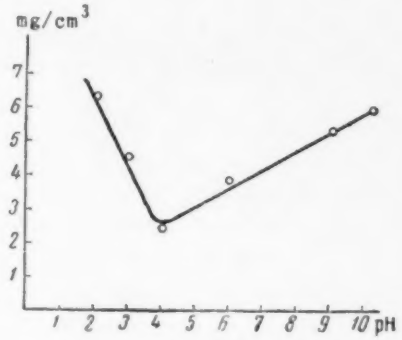


FIG 3. Curve of solubility of RNA.

The investigations made by both methods gave concurrent results; the isoelectric point was at a pH value of about 4·3, which in turn corresponds to the findings in the literature [6].

Figure 3 shows the solubility curve where the minimum solubility gives the isoelectric point of the RNA preparation.

These remarks may be considered as confirmation of the hypothesis previously advanced on the rôle of the orderly arranged polar ionic groups, forming a general molecular electric field, in the appearance of anomalous magnetic properties of nucleic acids.

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# PROBLEM OF THE POLYPHASE NATURE OF COLLAGEN*

A. L. ZAIDES

Central Research Institute of the Leather-Footwear Industry, Moscow

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VOL. 5 1960

A NUMBER of studies on the structure of collagen fibrils indicate their polyphasic nature and the multi-component system of collagen [1]. A fibril is the smallest structural unit of collagen and, therefore, in speaking of collagen we have in mind precisely this structural unit.

Repeated treatment of the dermis of young, small experimental animals (rats guinea-pigs) with citrate buffer at pH 4 made it possible to seperate the collagen into two microphases — procollagen and "collastromin" — which differ in structure as determined by the method of wide-angle X-ray structural analysis, electron microscopy and histochemical staining.

In the X-ray diffraction pattern of collastromin there is no interference ring, characteristic of the collagen complex and procollagen, corresponding to the interplanar distance of 2·9 Å; the electron microscopic structure of collastromin also differs from that for procollagen and the entire collagen complex as a whole. The fact that procollagen possesses a structure similar to the collagen complex is to be explained by the fact that collastromin forms the core of the collagen complex and procollagen is located at the periphery of the fibril (it is assumed that the fibril is of cylindrical shape). In this case the structure of procollagen determined by means of electron microscopy may correspond to that of the entire collagen fibril. However, the diffraction pattern of collagen is the result of superimposing those of procollagen and collastromin. Evidence of the superimposing of two diffraction patterns is given by the determination of the relative values of the intensities of the rings corresponding to the interplanar distances at 2·9 and 4·6 Å for collagen and procollagen [2]. The ratio of intensities for collagen is 0·26 and for procollagen 0·30.

It is assumed that the collagen complex contains about 80 per cent collastromin and about 20 per cent procollagen. With such difference in the quantitative contents of procollagen and collastromin in the collagen complex the difference in the relative values of the intensities of the interference maxima for the collagen and procollagen seems at first sight insufficient to postulate summation of two diffraction patterns. However, analysis of the diffraction pattern obtained shows the validity of this conclusion. The point is that the relative values of the intensities are determined according to the interference rings of which one characterizes the crystalline state (2·9 Å) and

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the other indicates the disposition of the atoms characteristic of liquid bodies. The intensity for the complete Debye ring is determined [4]:

$$Im = \frac{N^2 e^4 \lambda^3 \gamma}{8m^2 c^4} \frac{1 + \cos^2 2\vartheta}{\sin\vartheta} PF^2,$$

where $e$ is the charge on the electron, $\lambda$, the wavelength, $\gamma$, the volume traversed by the X-rays, $m$ the mass of the electron, $c$, the velocity of light, $P$, the repeat factor, $F^2$, the structural factor incorporating the atomic and the heat factors, $N$, the number of dispersion centres per unit volume.

The intensity of dispersion by a single atom of fluid is determined by [5]:

$$Im = N\psi^2 E_s \frac{1 + \cos^2 2\vartheta}{2}$$

where $E_s$ is the intermolecular factor, $\psi$, the atomic factor. Comparison of both formulae revealed the interesting point that in the case of crystalline substances the intensity of scatter is proportional to the square of the number of dispersion centres and in the case of fluid is directly proportional solely to the number of scattering centres. This explains the minor differences in the relative values of the intensities of interference as between collagen and procollagen. In order to estimate the rôle of all the factors taken together, the following model experiment was run.

Procollagen, in the form of crystalline needles, was dissolved in dilute acetic acid and a film prepared from this solution. Sufficient acetic acid was used so that after drying the film did not contain electrolytes. Then a film was prepared from egg albumin (dissolved in water with air drying of the solution on a chlorvinyl base). Then, the films of both substances were so combined as to give a thickness of the procollagen film of only 1/5th of the thickness of the entire irradiated preparation. Such a ratio corresponds to a content of 20 per cent procollagen and 80 per cent egg albumin. It is known that in the egg albumin diffraction pattern there is no ring corresponding to the interplanar distance at 2·9 Å but an amorphous ring corresponding to a distance of 4·6 Å is present.

Determination of the relative values of the intensities of the rings from the diffraction patterns for the film of pure procollagen (obtained from the acetic acid solution) and the preparation obtained by combining the films of procollagen and egg albumin gave the following results:

| | $I$ rat. |
|---|---|
| Procollagen+egg albumin (1:4) | 0·226 |
| Procollagen | 0·243 |

Consequently, both elementary calculation and the model experiment show that the diffraction pattern of collagen is the result of summation of two patterns.

Seperation of collagen into two structurally differing proteins was effected after repeated extraction of the comminuted tissue with citrate buffer. It could be assumed

that such prolonged treatment of tissue would lead to denaturation of the residue. Such statements are to be found in the literature. Thus, Harkness, Marko, Muir and Neuberger [6] showed that on successive fractionation with citrate buffer a gradual reduction in the content of procollagen is observed in individual fractions. Extrapolation of the figures to an indefinite number of extractions led these authors to assume that the amount of acid-soluble collagen as a percentage of the entire collagen must be about 20 per cent. The progressive fall in the procollagen content in the extracts may serve as an indication that conversion from a residue not soluble in citrate buffer to procollagen passing into solution is discontinuous, i.e. the notion of the polyphasic nature of collagen. However, these workers ascribe the reaching of the solubility limit in extraction of procollagen to protein denaturation. The same con-

VOL.
5
1960

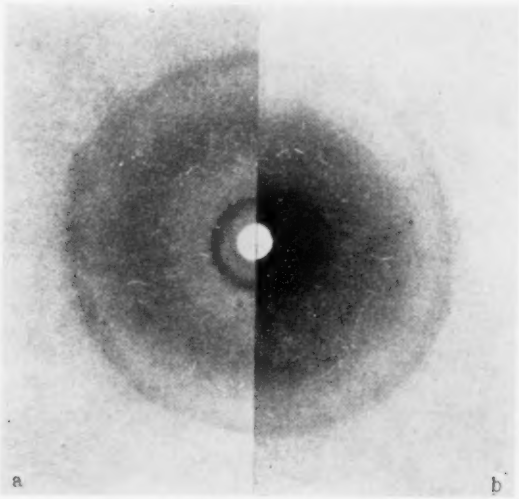


FIG. 1. (a) X-ray diffraction pattern of procollagen; (b) pattern of procollagen recrystallized 12 times.

clusion was reached by Grassmann [7]. He considers that the change in the diffraction patterns at large angles observed for collastromin is due to denaturation brought about by the citrate buffer. In the view of Grassmann, the difference between the soluble and insoluble part of collagen is that in the insoluble part there are transverse bonds not present in the soluble fraction.

If we start from the assumption that repeated citrate treatment causes denaturation of the residue then the same denaturation should also occur on repeat treatment of procollagen with citrate buffer. For this purpose we carried out an experiment in which procollagen was precipitated 12 times from citrate solution. The diffraction pattern of this residue after recrystallization (12 times) showed the presence of a ring corresponding to a distance at 2·9 Å. A higher degree of diffuseness was obtained for distances corresponding to the transverse bonds in the structure of the collagen

fibre (Fig. 1). Hence, repeat citrate treatment does not cause the disappearance of the interference ring characteristic of procollagen, corresponding to the interplanar distance, at 2·9 Å and consequently, the differences between the structure of procollagen and collastromin are not related to the denaturing effect of the citrate buffer.

Finally, further mention must be made of the problem of the embryogenesis of collagen. Our experiments [8] showed that during embryogenesis initially threads of collastromin are formed which as embryonic development proceeds, join the procollagen forming a system which we call collagen. These notions were confirmed by the X-ray structural method of analysis (absence of a ring corresponding to the interface distance at 2·9 Å). However, the electron microscope pattern differed somewhat from electron microphotographs of the collastromin threads. Whereas the col-

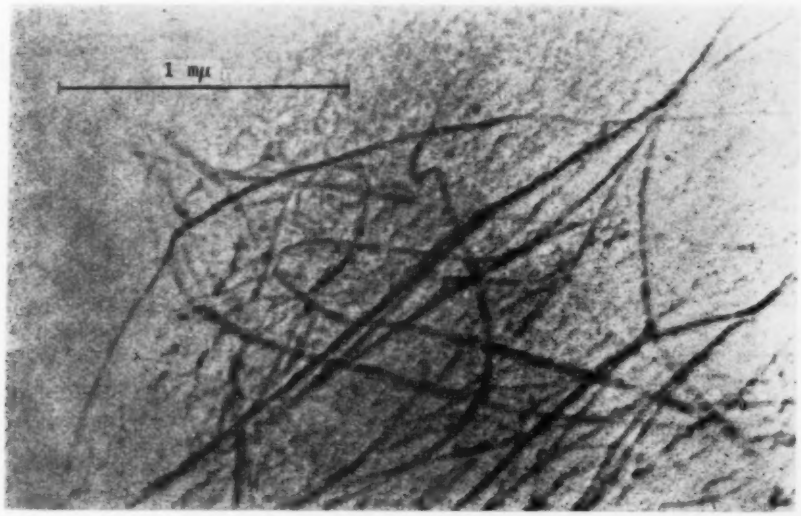


FIG. 2. Electron microphotograph of residue of collagen first treated with sulphite, then fractionated with citrate buffer.

lastromin threads are structureless, the embryonic fibrils displayed striations with a repeat period at about 280 Å. We ascribe these differences to the fact that the treatment applied in separation of the collagen complex made it possible to observe the collastromin threads by passing the stage of the collastromin fibrils. We have now demonstrated this supposition experimentally.

As we have already shown [2], preliminary treatment of connective tissue may exert an effect on the subsequent fractionation by citrate buffer. Thus, separation of collagen from the dermis of cattle (cow) into different microphases could be achieved only after preliminary treatment of the comminuted tissue with the fungus *Aspergillus nidulans* in the presence of sodium sulphite.

Preliminary treatment of the collagen of the comminuted calf dermis with sodium sulphite alone (1 M solution) made it possible during subsequent fractionation of

this tissue with citrate buffer at pH 4 to detect in the residue together with the threads, a large number of fibrils with a repeat period of the order of 280 Å (Fig. 2). The width of the fibril was of the order of 500 Å. In the original calf not subject to any treatment, such fibrils were not detected. This experiment entitles us to speak of the existence in collagen of collastromin fibrils with a structure detected by us in embryonic material. These results are in agreement with the observations of Porter and Vanamel [9] according to whom in tissue cultures, fibrils were detected with a repeat period of 270 Å which at the ends were split into individual fine threads.

Consequently, there is complete agreement between the findings obtained in the investigations on embryonic tissue and the method of fractionation of the dermis of already formed tissue.

The results provide some additional information on the structure of collagen.

The smallest structural unit of collagen is the fibril consisting of two microphases — procollagen and collastromin.

We had earlier assumed that collastromin consists of thin non-striated threads with a diameter of up to 100 Å. The experiments with preliminary treatment with sulphite have shown that collastromin threads are combined in collastromin subfibrils with a diameter of about 500 Å with transverse striations at about 280 Å. On formation of the collagen fibril procollagen apparently interacts not with the collastromin threads but with collastromin subfibrils.

OL.
5
960

## SUMMARY

(1) In investigation of the structure of collagen as a polyphasic formation we demonstrated in model experiments that: (a) the X-ray diffraction pattern of collagen is the result of summation of two diffraction patterns — procollagen and collastromin; (b) the differences in the structures detected by the method of X-ray structural analysis between the two microphases of collagen are not due to the denaturing effect of the citrate buffer used for seperating the collagen into the microphases.

(2) Investigation of the effect of preliminary treatment with sodium sulphite with subsequent fractionation of collagen of the calf dermis demonstrated the existence of collastromin fibrils with transverse striation at about 280 Å. Hence, it may be assumed that the collastromin threads earlier detected by us are aggregated into larger structural units — collastromin subfibrils which, on further interaction with procollagen, form collagen fibrils.

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# RELATION BETWEEN ELECTRICAL POLARIZATION AND RHYTHMICAL ACTIVITY OF STRIATED MUSCLE FIBRE*

VOL
5
196

P. G. KOSTIUK and A. I. SHAPOVALOV

Bogomol'ts Institute of Physiology, Academy of Sciences of the Ukrainian S.S.R., Kiev

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THE rhythmical activity developing in the striated muscle fibres in certain conditions and singularly resembling the natural rhythmical activity of cardiac fibres is quite a complex phenomenon. Its appearance is independent of the value of the membrane potential of the cell and is determined by more complex changes in the cell membrane and by processes of ion transport across the membrane [1–2]. However, steady rhythmical activity is extremely closely related to the level of electrical polarization of the cell surface frequency of the rhythmical oscillations increases with reduction in the membrane potential and decreases with a rise in the potential [2]. As yet, there is only fragmentary information, obtained from changes in potential resulting from various chance factors, available on the relation between rhythmical activity and the value of the membrane potential. The present work is devoted to an experimental study of the correlation between the frequency of the rhythmical activity and the membrane potential of the fibre. Two-channel intracellular microelectrodes were used. One of the channels of the microelectrodes served for recording the potential of the fibre, the other for polarizing its surface.

Experiments were carried out on frog sartorius muscles placed in Ringer solution with a reduced amount of calcium. The muscles were secured isometrically with stretching optimum for the appearance of rhythmical activity [2].

## METHODS

The two-channel microelectrodes were prepared from double-walled glass tubes by extruding them in an automatic apparatus [3]. In order to increase the mechanical stability of the micro-electrode (which is particularly important on work on muscle

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fibres with a quite tough surface) particularly strong types of glass (Pyrex) were used. The microelectrodes were filled with 2·5 M KCl by the method of Tasaki; use of a somewhat reduced concentration of electrolyte lessened the chances of crystallization within the electrode. As a rule, the resistance of the channels was not uniform and varied within limits 5–20 MΩ. Use of high-resistance electrodes was undesirable since in this case there is a substantial increase in the artefacts on making and breaking the current, associated with the capacitive interaction of the channels. Electrodes with a resistance of 6–10 MΩ proved most convenient. One of the channels of the electrode was connected to the input stage of an amplifier (cathode follower) by means of a short flexible chlorinated silver wire introduced directly within the channel. The second channel by means of similar wire was attached to the polarization system. The possibility of short circuiting between the channels at the site of immersion of the wires was avoided by withdrawing electrolyte from the upper part and by careful drying of the glass surface. For the recording and polarization system separate extracellular electrodes, also in the form of chlorinated silver wires, were used; the extracellular electrode in the polarization system was attached as close as possible to the fibre under investigation.

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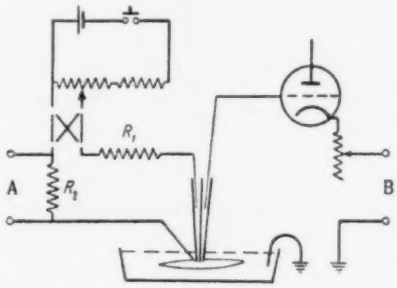


FIG. 1. Arrangement for polarization and recording potentials of muscle fibre by means of two-channel intracellular microelectrode. $R_1 = 70$ MΩ; $R_2 = 300$ kΩ.

Polarization was effected according to the scheme illustrated in Fig. 1; the strength of the polarizing current was determined by measuring the drop in voltage over resistance $R_2$. The potential at the ends of this resistance was amplified by a d.c. amplifier (A) and recorded on one beam of a two-beam cathode oscillograph; on the second beam the membrane and spike potentials of the fibre were recorded, amplified by means of a second d.c. amplifier (B).

Difficulties arising in the use of two-channel intracellular microelectrodes have already been discussed in the literature [4]. They consist of the total resistances in the polarizing and recording circuits which distort the true value of change in membrane potential and the presence of capacitive coupling between the channels leading to considerable artefacts at the moment of making and breaking the polarizing current. The total resistances, apart from the resistance of the cell membrane, are the resistance of the medium surrounding the fibre (from the cell surface to the relevant electrode)

and the resistance of the myoplasm (from the tip of the microelectrode to the inner surface of the cell membrane). Experience shows that the total resistance of the segment in the direct vicinity of the tip (and perhaps at the very tip itself) is particularly high; the resistance of the myoplasm itself and the external solution (where the extracellular electrode is located in the direct proximity of the cell surface) is insignificant compared

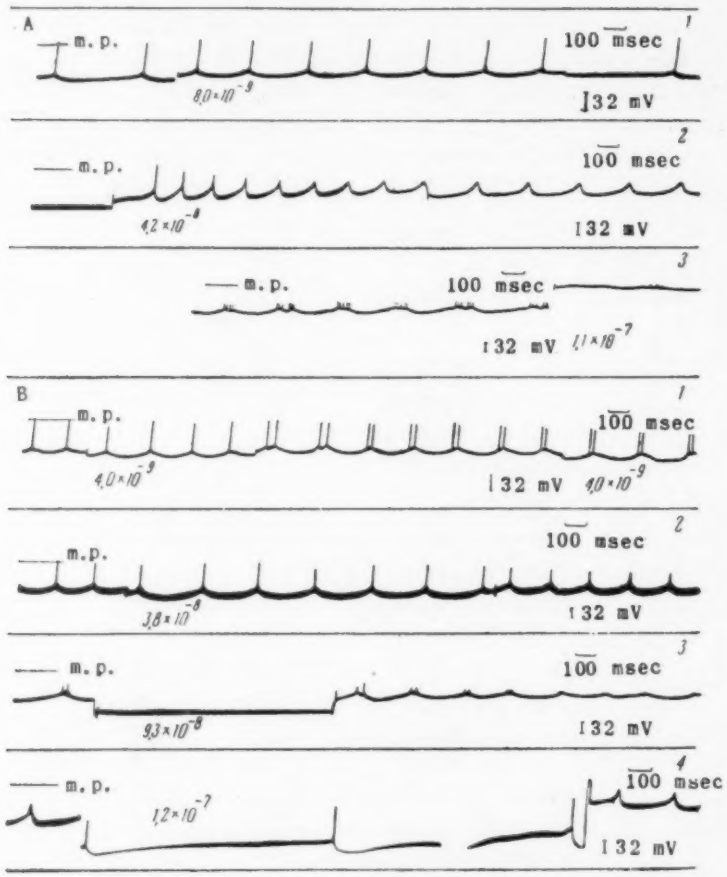


FIG. 2. Examples of changes in rhythmical activity of muscle fibres on depolarization (A) and hyperpolarization (B).

to the total resistance at the tip of the microelectrode and the resistance of the membrane. Therefore, the true value of the drop in potential over the resistance of the membrane may be determined as follows. The drop in potential on passing a known polarizing current is determined twice, on positioning the microelectrode within the cell and on positioning it outside directly adjacent to the cell surface. The value obtained in the second case was less and the difference in these values chiefly reflects the drop in potential over the cell membrane. Of course, such a calculation is not particularly

accurate. It is based on the assumption that the total resistance at the tip of the micro-electrode does not change when the micro-electrode penetrates the cell. However, some change in this resistance is quite probable. In our oscillograms a correction was made in order to establish a more precise value of the change in the membrane potential. Polarization is characterized by the values of the current; since the value of the external resistance in the polarizing circuit ($R_1$) is very high as compared with the resistance of the cell and the tip of the microelectrode, the variations in the latter made scarcely any difference to the value of the polarizing current and to all intents and purposes a constant current was achieved. Since in our experiments, relatively slow developing processes and prolonged polarization were studied, the short-lived distortion on making and breaking the current, associated with the capacitive coupling between the channels, was slight. Therefore, we did not use any special measures to reduce it.

VOL. 5 1960

## RESULTS

A change in the potential of the membrane of the muscle fibre usually did not cause the appearance of rhythmical activity in "quiescent" fibres. This confirms the conclusion that reduction in the value of the membrane potential (m.p.) as such is not a factor inducing rhythmical activity [2]. However, in individual muscle fibres it is possible to observe the appearance of rhythmical activity after complete depolarization. But here too, it was easy to see that the rhythmical activity did not directly depend on depolarization and the latter was only the impetus for fibres predisposed to such activity. While rhythmical activity developed under the influence of depolarization, return restoration of the m.p. of the fibre to the original level did not stop the rhythmical activity (Fig. 2 (A), oscillogram 2).

However, change in the polarization of the fibre against the background of the existing rhythmical activity caused strictly constant and regular changes in the latter. Reduction in the value of the m.p. was always accompanied by an increase in the frequency of the rhythmical activity; increase in the m.p., by a reduction in its frequency. Appreciable changes in the frequency of the rhythmical activity developed on polarization with currents of about $0{\cdot}5\times10^{-8}$ A which produced a shift in the m.p. only by several mV, considerably less than the threshold changes for direct excitation of the muscle fibre. Extremely strong hyperpolarization completely arrested rhythmical activity, this occurred for currents of the order of $5\times10^{-8}$–$9\times$ (although in one case rhythmical activity with an exceptional rhythm, persisted even on hyperpolarization of the fibre with a current of $1{\cdot}2\times10^{-7}$ A). With strong depolarization, switching on the current was accompanied by exscitation in the form of one spike potential (s.p.) or a rhythmical discharge; the rhythmical activity was disturbed and it was impossible to determine its frequency.

Figure 2 shows oscillograms illustrating the changes in the rhythmical activity on depolarization (A) and hyperpolarization (B) of a muscle fibre. In oscillogram I (Fig. 2 (A)) and oscillogram 2 (Fig. 2 (B)) examples of change in the existing rhythmical activity are given; in oscillogram 2 (Fig. 2 (A)) appearance of rhythmical activity

in the "quiescent" muscle fibre is shown, oscillograms 3 show examples of the effect of strong hyperpolarization and depolarization leading to complete removal of rhythmical activity. Oscillogram 4 (Fig. 2 (B)) represents a case of preservation of rhythmical activity with a particularly rare rhythm on very strong hyperpolarization.

It was impossible to plot the curve of the relation between the change in the membrane potential and change in the rhythmical activity frequency in a single fibre, since in each fibre the rhythmical activity lasted for a relatively short time and we were unable to follow the effects due to polarization by currents of varying strength and varied direction during this period. Successive measurements on the same fibre were rendered difficult also by the fact that the frequency of the rhythmical activity in this fibre rapidly fell. Therefore, we made a comparison of the changes in the rhythmical activity on polarization of different fibres with currents of different direction and strength. Since the frequency of the rhythmical activity in different fibres varied considerably, it was impossible to compare absolute values. But on comparison of the relative values (frequency of rhythmical activity for a given polarization in relation to the rhythmical activity frequency before switching on the polarizing current) an extremely strict relationship was obtained between rhythmical activity and the polarizing current. The relevant graph plotted from measurements in fifteen muscle fibres is presented in Fig. 3.

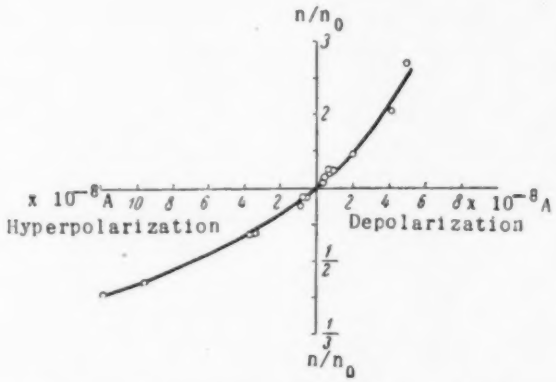


FIG. 3. Curve of relationship between strong polarizing current and change in relative frequency of rhythmical activity.

Direct variation of the m.p. permits more precise analysis of the causes for the developing changes in rhythmical activity. A particularly good way to do this is to superimpose individual cycles of the rhythmical activity obtained against a background of unchanged and changed m.p. In the majority of fibres the slow depolarization ("prepotential") slowly rose at first, then its slope became increasingly steep and ultimately passed without any noticeable break into the usual spike potential (s.p.). Sometimes, however, the prepotential was quite distinctly separated from the origin of the s.p. by a steep bend. Apparently, the differences in the shape of the potential

are associated with the location of the point of generation of the s.p. and the microelectrode. Continuity was characteristic when recording directly from the site of generation of the s.p.; the distinct separation of the prepotential from the s.p. was observed on recording at a certain distance from this site — where the s.p. arrives after a definite delay — as compared with the electrotonic spread of the slow oscillation. Such an explanation of these differences was proposed for example, by Weidmann [5] for passage of the prepotential into a s.p. in the pacemaker of the heart. Obviously, in the first case a more accurate definition of the effect of the various components in the change of the rhythmical activity on polarization is possible.

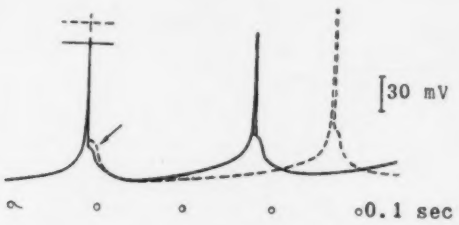


FIG. 4. Comparison of two cycles of rhythmical activity obtained for same fibre on changing its membrane potential by 20 mV. Horizontal lines indicate value of membrane potential in both cases.

Figure 4 shows an example of the comparison of the rhythmical activity cycles obtained before and immediately after change in the m.p. A very conspicuous feature is the basic rôle of the slope of the rise in the prepotential (in particular, of the slowly developing portion) in determining the rhythmical activity frequency. With an increase in the m.p. prepotentials developed more gradually, with a reduction, more rapidly.

When depolarization reached a particular level, the subsequent process showed little dependence on the m.p. Because we could not establish precisely the position at which the prepotential ended and where its actual peak commenced we were unabl to establish any changes in the critical level of the prepotential at which generatione of the s.p. occurs. There is little point in using for this purpose cases with distinct separation of the ascending portion of depolarization, since they do not reflect the effective ratios between the prepotential and the s.p. at the site of generation of the latter. Some conclusions may be drawn from observation of the descending portion of a s.p. displaying a distinct bend which possibly represents the apex of the prepotential. In any case, it cannot be considered as after-depolarization following the s.p., since it persisted also after reduction in the spike by an inconsiderable local change (see below). It may be noted that for a high m.p. this bend was somewhat higher as compared with the initial level of the m.p. than for a low m.p. (arrow in Fig. 4); apparently, with a rise in the m.p. the prepotential ought to reach a high value in order to generate a s.p. (excitability of the fibre is reduced). However, these changes were very slight and obviously do not play an important rôle in determining the rhythmical activity frequency.

Nevertheless, it should be noted that each cycle of maximum hyperpolarization at which rhythmical activity is still possible, retains all its components and always starts with gradual depolarization of the fibre membrane. Attenuation or disappearance of the prepotentials but with retention of the rhythmic s.p. were never observed. Thus, even the application of such a technique of investigation failed to confirm the assumption of Bulzring [1] that slow depolarization is the property solely of fibres with low membrane potentials. Generation of the s.p. for rhythmical activity in striated muscle fibres as in all other rhythmical active structures, is always associated with development of prepotentials.

It is natural that with change in the value of the m.p. there occur corresponding changes in the s.p. — on depolarization, the amplitude of the latter falls and the rate of rise is somewhat slowed down; on hyperpolarization the converse is true. With considerable depolarization the s.p. became smaller and finally disappeared. Nevertheless, the rhythmical prepotentials still continued to appear for quite a long time even with a further drop in the m.p. In fact, complete disappearance of the slow rhythmic oscillations of the m.p. occurred only when the latter fell almost to zero. After the disappearance of the typical s.p. a minute spike, frequently a double one, was usually visible at the apex of the prepotential. On the one hand, this could have been the expression of a local process of excitation in the region of the microelectrode. On the other, it is possible that the site of generation of the s.p. in such cases shifted further from the site of insertion of the microelectrode. The s.p. formed there could not spread to the area of the fibre with very reduced m.p. but caused only insignificant electrotonic changes in this area.

In the case of intensive hyperpolarization such dissociation between blocking of formation of a s.p. and the prepotentials was not observed.

Ease of generation of s.p. on depolarization and obstruction of it on hyperpolarization could be observed in certain fibres in a different form; while before switching on the current each prepotential generated one s.p., with depolarization a rhythmic discharge of s.p. appeared in each slow wave (as is usually the case in normal fibres with a particular predisposition to rhythmic activity [2]). On the other hand, when several s.p. were formed in each prepotential switching on a hyperpolarizing current reduced their number to one.

Distinct changes in rhythmical activity were observed after switching off the polarizing current; in this case they were opposite to those which occurred during polarization. Sometimes, such changes could be observed also after polarization with weak currents which in themselves caused only minor changes in rhythmical activity. After switching off a hyperpolarizing current it was possible to observe the appearance in each wave of the prepotential of two or several s.p. although before and during hyperpolarization each prepotential generated only one spike (oscillogram 1, Fig. 2 (B)); some increase in the frequency of rhythmical activity was possible, as compared with its frequency before switching on the current. After switching off weak depolarizing currents, changes of opposite nature were observed.

VOL
5
196

The considerably more distinct "off effects" in the rhythmical activity appeared on application of strong currents (of the order of $1 \times 10^{-7}$ A). The use of hyperpolarizing current of such strength in the "quiescent" cell produced in a number of cases subsequent stable rhythmical activity and in the rhythmically active cell a well-marked

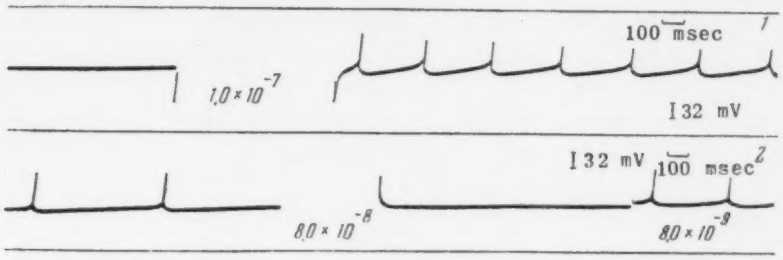


FIG. 5. Effect of switching off strong hyperpolarization (oscillogram 1) and strong depolarization (oscillogram 2) on rhythmical activity.

acceleration of rhythm. The use of depolarization on the other hand, inhibited the rhythmical activity and the m.p. had to be reduced to a certain level in order to generate it. The relevant examples are given in the oscillogram in Fig. 5. Indication of similar effects of switching off have also been given for rhythmically active neurones of the lobster heart ganglion [6].

## DISCUSSION

The results confirm the findings previously obtained by observing changes in rhythmical activity and m.p. of the muscle fibre due to various random factors. The rhythmical activity is based on a certain distinctiveness in the functioning of the surface structures of the cell (associated primarily with calcium ions) which is manifest in the most diverse conditions of the membrane potential. The main aspect of this phenomenon is a progressive fall in the m.p. (prepotential) which is increasingly accelerated and finally passes into the usual spike potential. It is assumed from the fact that the generation of rhythmic prepotentials is suppressed on removal of sodium ions from the surrounding medium [2] that their origin and also that of the s.p. involves movement of sodium ions across the surface of the cell along a concentration and electrochemical gradient. Such an assumption has also been advanced in relation to the mechanism of slow depolarization in the pacemaker of the heart [7]. That the mechanisms of generation of prepotentials and the s.p. are linked is also indicated by the presence in both cases of opposite effects on switching on and off an electrical polarizing current (although in respect of threshold values, between the effect on the course of the prepotentials and the direct excitatory effect on the fibre, there is a fundamental difference).

However, a considerable difference does exist between the prepotential and the s.p. The slope of the rise of both depends on the value of the m.p., but if the slope of the spike increases within definite limits on hyperpolarization of the membrane (as ought to be the case if it is due to the movement of ions along the electrochemical

gradient) the slope of the rise of the prepotential (at least its initial portion) distinctly diminishes. It follows merely from this that the mechanisms of depolarization of the cell surface cannot be identical in both cases.

The fact that on considerable depolarization of fibres the s.p. rapidly disappear and the prepotentials continue to be recorded (in fact the m.p. can be reduced almost to zero), makes the latter to some extent similar to "post-synaptic potentials", in particular, to the potentials of the end plate. A similar gradual decrease with reduction in the m.p. to zero has led to the assumption that the potentials of the end plate are associated not with the appearance of specific permeability of the membrane to sodium ions, as in the case with generation of s.p., but with an unspecific rise in the permeability to a series of ions [8, 9].

However, there are also considerable differences between the "post-synaptic potentials" and prepotentials. The former on hyperpolarization of the membrane within certain limits have an increased rate of rise as has been shown for muscle fibres [8] and motor neurones [10]; the latter have a reduced rate. If the prepotentials were associated, as are the post-synaptic potentials, with a rise in the permeability of the membrane to a number of ions, then as they develop, resistance of the membrane ought to fall [9]. For the rhythmical activity of striated muscle fibres such measurements have not been made but for the fibres of the pacemaker of the heart it has been shown that in this period the resistance of the membranes, on the contrary, gradually rises and only at the moment of passage of the prepotentials into a spike, does it sharply fall [11].

It must therefore be recognized that in the prepotentials we have an expression of a particular form of excitatory process with a special mechanism, differing from that of the spreading excitation associated with the spike potential and the local processes of excitation both of synaptic and non-synaptic origin manifest in the post-synaptic potentials in abortive spikes.

A striking point is the extremely great similarity between the changes in rhythmical activity induced in striated fibres by artificial change in the polarization of their surface to the natural change in rhythmical activity of the heart pacemaker under the influence of parasympathetic and sympathetic stimulation. In the heart also, one of the basic factors in reduction of rhythms is retardation or acceleration of the rise in prepotentials [12, 13]. The vagus effect is usually combined with hyperpolarization of the membrane; the sympathetic effect is not associated with a fundamental change in the initial level of the m.p. Apparently, the development of the cellular processes underlying the prepotentials can be controlled both by change in the potential of the membrane and by bringing humoral factors to bear on them; thus, these processes turn out to be a universal mechanism of control of rhythmic activity.

## SUMMARY

(1) Two-channel intracellular microelectrodes have been used to investigate the relationship between the value of the membrane potential and the rhythmical activity of muscle fibres of the frog sartorius muscle. The rhythmical activity was induced

by a reduction in the concentration of calcium ions in the surrounding solution. One of the channels of the microelectrodes served to record the potentials of the fibre, the other, to polarize its surface with incoming or outgoing currents.

(2) Increase in the membrane potential was accompanied by a reduction in the frequency of rhythmical activity and reduction by an increase in the frequency. The threshold changes in rhythmical activity developed on polarization with a current of about $0{\cdot}5\times10^{-8}$ A. Hyperpolarization with a current of the order of $1\times10^{-7}$ A completely suppresses rhythmical activity; strong depolarization of the fibre disturbs rhythmical activity in connexion with a direct excitatory effect.

(3) The main factor determining change in frequency in rhythmical activity was change in the slope of the rise of the prepotential — initial portion of the slow depolarization preceding generation of a spike potential. This is similar to the changes in the slope of the prepotential in tissues with natural rhythmical activity (heart pacemaker) under the influence of parasympathetic and sympathetic impulsation.

VOL. 5 1960

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* There is a translation into English of this work.

# DETERMINATION OF THE ANGULAR DISPLACEMENTS OF THE MEMBERS OF THE UPPER LIMB FROM THE CO-ORDINATES OF ITS JOINTS*†

V. Z. OSHEROVICH

Leningrad Prosthetic Appliances Research Institute

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VOL
5
196

THE movements of the limbs of man are made up of the rotations of their members at the joints and turning of these members about their longitudinal axes. Therefore, finding the angles with which at various times these rotations and turns are measured is one of the most important aspects of the investigation of human movements.

In the literature [1] this problem has been treated only in relation to the lower limb which, in performing its basic motor functions (walking and running), is displaced in one — the sagittal — plane. The manifold movements of the upper limb are nearly always three-dimensional, i.e. incomparably more complex. The present paper gives strict mathematical definitions of the angles characterizing the spatial movements of the upper limb (as distinct from the usual descriptive and imprecise definitions of these angles) and simple formulae are evolved for computing these angles from given co-ordinates of the joints of the upper limb.

Let the co-ordinates $x$, $y$ and $z$ of the joints be known from mirror cyclography i.e. the co-ordinates of; (1) shoulder $b$; (2) elbow $a$; (3) radiocarpal $m$; (4) point $g_m$ coinciding roughly with the central metacarpophalangeal joint of the hand; and (5) point $m_1$ siutated at a certain invariable distance from point $m$ perpendicular to the axis of the forearm in the direction of the bent back thumb (Fig. 1). The co-ordinates of these points vary, of course, with time by virtue of movement of the arm but at any given moment they fully determine its position in space.

In deriving the relations below, we assumed a position of the subject with respect to a fixed Cartesian system of co-ordinates related to the arrangement for cyclography so that the anatomical axes and planes of the trunk were parallel to the corresponding co-ordinate axes and planes. We assumed that as in Fig. 1 the longitudinal axis of the subject's trunk was parallel to the co-ordinate axis $Y$, the antero-posterial to axis $Z$

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† Read to the scientific session of the Laboratory of Physiology of Labour Processes of the Ukhtomskii Physiology Institute of the Leningrad State University (4 November 1955) and to the section of Labour Physiology and Physical Education of the Leningrad Sechenov Society of Physiologists, Biochemists and Pharmacologists (10 April 1956).

and the transverse to axis $X$. Then, the frontal plane of the trunk will be parallel te the co-ordinate plane $XOY$, the sagittal to plane $YOZ$ and the transverse to plane $XOZ$.

We shall denote the length of the upper arm, i.e. the distance between points cyclography lamps) $b$ and $a$ by $L_1$ and the length of the forearm or distance am by $L_2$. The distances $mg_m$ (arbitrary length of the wrist) and $mm_1$ (arbitrary length of the thumb) correspond respectively to $L_3$ and $L_4$.

It is obvious that:

$$\left.\begin{aligned} L_1 &= \sqrt{(x_b - x_a)^2 + (y_b - y_a)^2 + (z_b - z_a)^2} \\ L_2 &= \sqrt{(x_a - x_m)^2 + (y_a - y_m)^2 + (z_a - z_m)^2} \\ L_3 &= \sqrt{(x_m - xg_m)^2 + (y_m - yg_m)^2 + (z_m - zg_m)^2} \\ L_4 &= \sqrt{(x_m - x_{m1})^2 + (y_m - y_{m1})^2 + (z_m - z_{m1})^2} \end{aligned}\right\} \quad (1)$$

(1) The angle $\alpha$ of abduction-adduction in the shoulder joint characterizes the rotation of the upper arm about the antero-posterior axis drawn through the centre of this joint. This angle is formed by the projections of the axis of the upper arm in its original and given (the one under consideration) positions in a plane perpendicular to the axis of rotation, i.e. frontal. Since, in the starting position the upper arm is parallel to the longitudinal axis (freely hanging arm when standing upright), then the angle in question is enclosed between axis $Y$ and the projection on the frontal plane $XOY$ of the axis of the upper arm $ba$ in its given position (Fig. 2).

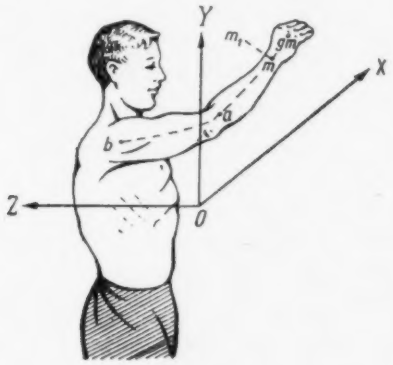


FIG. 1.

It thus follows that the angle may be found from the relation:

$$\tan \alpha = \frac{x_b - x_a}{y_b - y_a} \quad (2)$$

(2) The angle $\beta$ of flexion-extension in the shoulder joint characterizes the rotation of the upper arm with respect to the transverse axis of this joint.

This angle is measured in the sagittal plane and is equal to the angle formed by the projection of the axis of the upper arm in the given plane with the axis $Y$ (Fig. .2) Like the preceding angle it can be expressed by:

$$\tan\beta = \frac{z_b - z_a}{y_b - y_a} \tag{3}$$

(3) The angle $\gamma$ between the axes of the upper and forearm is called the angle of flexion of the elbow joint.

The determine it we shall consider a triangle *bam* formed by the upper and forearm and the straight line connecting the shoulder and radiocarpal joints *bm* (Fig. 2). In this triangle the lengths of the two sides: upper ($L_1$) and forearm ($L_2$) are already known (formula 1) and the length of the third side *bm* is computed from the co-ordinates of the apices *b* and *m*:

$$(bm)^2 = (x_b - x_m)^2 + (x_b - y_m)^2 + (z_b - x_m)^2. \tag{4}$$

Applying the cosine law, we find:

$$\cos\gamma = \frac{L_1^2 + L_2^2 - (bm)^2}{2L_1L_2}. \tag{5}$$

Since, on movement of the upper limb only the distance *bm* can change and not the values $L_1$ and $L_2$ which are dependent on the individual characteristics of the subject, then formula (5) may be written in the form:

$$\cos\gamma = \frac{E - (bm)^2}{F}, \tag{6}$$

where $E = L_1^2 + L_2^2$ and $F = 2L_1 L_2$ are constants.

(4) Turning of the upper arm around its longitudinal axis is called rotation of the upper arm.

In the absence of rotation, the forearm ought to be located in plane I passing through the axis of the upper arm perpendicular to the frontal plane, i.e. inclined at the angle $\alpha$ of abduction to the sagittal. Through rotation of the upper arm it comes out of this plane. We shall denote by the numeral II the plane in which the upper arm and forearm lie, i.e. drawn through points *b*, *a* and *m*. Then, the angle of rotation $\delta$ is determined as the dihedral angle between planes I and II.

In order to find this angle, from point *m* we shall draw two perpendiculars; *mc* to the axis of the upper arm *ba* and *md* to plane I, the points *c* and *d* joined by a straight line (Fig. 2). The axis of the upper arm *ba* is the line of intersection of the planes I and II, i.e. the apex of the required dihedral angle $\delta$. The straight lines *cd* and *cm* forming the angle (first in plane I, second in plane II) are perpendicular to *ab*. Therefore, the angle of rotation $\delta$ is measured by the plane angle *mcd* (Fig. 2).

In the right-angled triangle *mcd*, the hypotenuse $mc = L_2 \sin\gamma$ (from $\Delta amc$; *mc* perpendicular to *ba*, by construction) while the line *md* is equal to the distance from

point $m$ to plane I. As is known from analytical geometry, the distance $h$ from the point $(x_0, y_0, z_0)$ to a plane $Ax+By+Cz+D=0$ is given by the formula:

$$h = \frac{Ax_0 + By_0 + Cz_0 + D}{\sqrt{A^2 + B^2 + C^2}}. \tag{7}$$

Plane I is represented by the equation:

$$(x - x_b)(y_b - y_a) - (y - yb)(x_b - x_a) = 0. \tag{8}$$

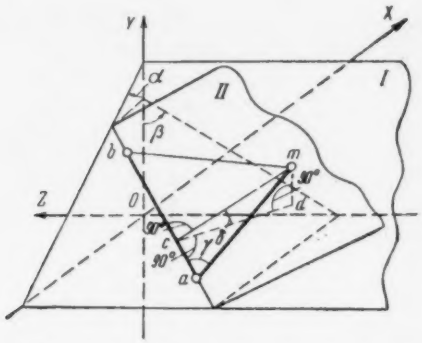


FIG. 2.

Substituting in (7) instead of the coefficients $A$, $B$, $C$ and $D$, their values from (8) and instead of $x_0$, $y_0$, $z_0$, the co-ordinates of the point $m$, we get the length of the segment $md$:

$$md = \frac{(x_m - x_b)(y_b - y_a) - (y_m - y_b)(xb - x_a)}{\sqrt{(x_b - x_a)^2 + (y_b - y_a)^2}}.$$

Noting that $\sqrt{(x_b - x_a)^2 + (y_b - y_a)^2}$ is the projection of the length of the upper arm on the frontal plane $XOY$, while

$$\frac{x_b - x_a}{\sqrt{(x_b - x_a)^2 + (y_b - y_a)^2}} = \sin\alpha, \text{ a } \frac{y_b - y_a}{\sqrt{(x_b - x_a)^2 + (y_b - y_a)^2}} = \cos\alpha$$

(see also formula (2)), we get:

$$\sin\delta = \frac{md}{mc} = \frac{(y_b - y_m)\sin\alpha - (x_b - x_m)\cos\alpha}{L_2 \sin\gamma} \tag{9}$$

(5) The angle $\varepsilon$ of flexion-extension in the radiocarpal joints is the angle between the axes of the forearm and the wrist (carpus $mg_m$).

The angle $amg_m$ being considered can, in like manner to the elbow, be given by the following formula:

$$\cos\varepsilon = \frac{G-(ag_m)^2}{H}, \tag{10}$$

where $G = L_2^2+L_3^2 = \text{const.}$ and $H = 2\,L_2\,L_3 = \text{const.}$ while

$$(ag_m)^2 = (x_a - x_{g_m})^2+(y_a - y_{g_m})^2+(z_a - z_{g_m})^2.$$

(6) Turn of the forearm about its longitudinal axis is called pronation or supination depending on whether it is performed inwards or outwards.

Like the angle of rotation, the angle of pronation-supination $\xi$ is defined as the dihedral angle between the plane of the upper arm and the forearm II and plane III (not shown in Fig. 2) passing through the axis of the forearm $am$ and the segment $mm_1$, i.e. through points $a$, $m$ and $m_1$.

Since, the segment $mm_1$ lying in plane III is perpendicular to the axis of the forearm $am$ along which planes II and III intersect, the dihedral angle $\xi$ betwen them is equal to the angle formed by segment $mm_1$ with the plane II. Knowing that the distance $h_1$ from the point $m_1$ to plane II is equal to:

$$h_1 = \frac{P(x_m - x_{m1})+Q(y_m - y_{m1})+R(z_m - z_{m1})}{\sqrt{P^2+Q^2+R^2}}$$

and knowing the constant length $L_4$ of the segment $mm_1$ from formula(1) we find

$$\sin\xi = \frac{h_1}{mm_1} = \frac{P(x_m - x_{m1})+Q(y_{m1} - y_{m1})+R(z_m - z_{m1})}{L_4\sqrt{P^2+Q^2+R^2}}, \tag{11}$$

where $P$, $Q$ and $R$ the coefficients for $x$, $y$ and $z$ in the equation for plane II having the values:

$$\left.\begin{aligned} P &= (y_b - y_a)(z_b - z_m) - (y_b - y_m)(z_b - z_a) \\ Q &= (x_b - x_m)(zb - z_a) - (x_b - x_a)(zb - z_m) \\ R &= (x_b - x_a)(y_b - y_m) - (x_b - x_m)(y_b - y_a) \end{aligned}\right\} \tag{12}$$

The validity of the relations derived has been verified experimentally on a mechanical model of the upper limb constructed in the laboratory of biomechanics of the Leningrad Prosthetic Appliances Research Institute. To speed up calculations of the usual mass character nomograms may be used for these relations.

In conclusion it should be noted that these formulae may also be applied for analysis of complex (for example, certain athletic) movements of the lower limb when the relative displacements of the adjacent members is not merely a matter of rotations about transverse axes of the corresponding joints.

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# ABILITY OF CHLOROPHYLL TO PHOTOSENSITIZE REDOX REACTIONS IN THE ADSORBED STATE*

V. B. YEVSTIGNEYEV and V. A. GAVRILOVA

Bakh Institute of Biochemistry, U.S.S.R. Academy of Sciences, Moscow

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VOL. 5 1960

RECENT findings obtained by electron microscopy [1, 2] and a number of physical methods of investigation [3] indicate that chlorophyll is arranged in the chloroplast granules in the form of thin, perhaps monomolecular, layers separated by layers of proteins and lipoids. On the other hand, it has long been postulated, a postulate supported by experimental research, that it is not the free molecules of the pigment which are active in photosynthesis but complexes with proteins and lipoids. The instability of such a complex, readily breaking down on exposure to various factors, suggests that the link between the pigment and its carrier is accounted for not by genuine chemical but rather by adsorption forces.

Therefore, study of the properties of chlorophyll and, in particular, its ability to photosensitize reactions in the adsorbed state in model systems is of considerable interest. However, there is an almost complete dearth of experimental material on this problem and it certainly cannot be regarded as settled.

Together with numerous findings on the photosensitizing action of chlorophyll in solution [4, 5] information has now been gained indicating that also in the aggregated state in the form of colloids [6, 7] and suspensions [8, 9] this pigment is able to sensitize redox reactions to light absorbed only by the pigment sensitizer.

In our previous communication [9] it was assumed from the experimental findings obtained with chlorophyll in the molecular-disperse state [10] and measurements of the photopotentials of pigment films [11], that the sensitizing action of the pigment particles is due to formation of surface reduced ion radicals of the pigment which then react with an electron acceptor. It may thus be expected that in the adsorbed state, for adsorbents which leave free or at any rate reactive the basic porphyrin part of the chlorophyll molecule, the latter will show photosensitizing action.

This work is concerned with the ability of chlorophyll and other pigments of similar structure to sensitize when in the adsorbed state.

## MATERIAL AND APPARATUS

Chlorophyll *a* and *b* were preapared by chromatography of their mixture obtained by the method of Zscheile [12] from dried nettle powder on a sucrose column.

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Protochlorophyll was extracted from the inner membranes of pumpkin seeds by the method described in paper [13]; bacteriochlorophyll from the purple bacteria *Rhodopseudomonas (Athiorodaceae)* by the same method [14].

The pheophytins of these pigments were obtained by treatment of ethereal solutions of the pigments with acid and subsequent removal of acid by washing with water and chromatography on sucrose.

Magnesium and copper phthalocyanine were present in the form of crystalline powders.

The solvents — ethanol, methanol, dioxane, acetone, etc. — from which adsorption was effected were the usual pure reagents not subjected to any special purification and used in the work after simple distillation. The powders used as adsorbents were also not given special treatment.

The absorption was measured in a quartz Beckmann spectrophotometer DU in special vacuum vessels [10] or in conventional tubes if anaerobic conditions were not called for. The air was evacuated for 3 min by a vacuum pump.

The source of illumination was a 30 V, 400 W incandescent lamp plus condenser. In the experiments at room temperature, the part of the vessel for illumination was placed in a jacket to prevent access of extraneous light to the solution. In the experiments at reduced temperature a Dewar vessel with transparent walls was used and to remove the unwanted light from the illuminating beam a glass filter not passing infra-red rays and a filter separating out the requisite region of the visible spectrum were incorporated. In the case of chlorophyll KS-10 or KS-15 light filters not passing light rays of wavelength less than 610 and 640 mμ respectively were most often used.

## METHODS

We usually exposed to illumination a system consisting of a suspension of the adsorbate of the pigment in aqueous medium in which were dissolved the electron dono, and acceptor (which did not reacting between themselves in the dark). As donorr use was made of ascorbic acid and the azo dye methyl red, long used in study of the photosensitizing properties of chlorophyll [4], acted as acceptor. The progress of the sensitized reaction was followed by the reduction in the value of the absorption maximum of the acceptor; in the case of methyl red in acid medium in the 520 mμ region. The results of the changes given in the graphs are expressed in percentages of the value of the original extinction coefficient. The latter was determined by subtracting the value of the extinction coefficient, at the given wavelength, after complete decolorization of the acceptor from the value of the extinction coefficient at the start of the experiment. These calculations were necessary owing to the absorption and scatter of light at any wavelength by the suspension of the pigment adsorbate itself. In order to obtain comparative data, the measurements of absorption of the suspensions of pigment adsorbates at each point were made with a definite time lapse (30 sec) after agitation.

## RESULTS

Figure 1 shows the results of an experiment using powdered $Al_2O_3$ as adsorbent of the pigment chlorophyll. The adsorbate was obtained by adsorption from alcoholic solutions of chlorophylls *a* and *b* as follows; 0·2 ml concentrated solution of chlorophyll in alcohol was added to 0·2 g $Al_2O_3$ and the alcohol removed by washing with water in a glass filter. The adsorbate was then washed off the filter into a reaction vessel with 5 ml water and to it were added ascorbic acid (20–40 mg) and ~ 0·05 ml concentrated solution of methyl red in water containing a small amount of phosphate buffer (pH 8·3). The $K_{52}$ of the reaction mixture due to the methyl red was usually 0·6–0·8.

As the Figure shows, illumination with red light in the presence of the chlorophyll adsorbate produced quite rapid decolorization of the methyl red. In the controls with suspensions not containing chlorophyll and chlorophyll adsorbates without ascorbic acid even after prolonged illumination (15–30 min) there was scarcely any change in the absorption of the methyl red.

VOL. 5 1960

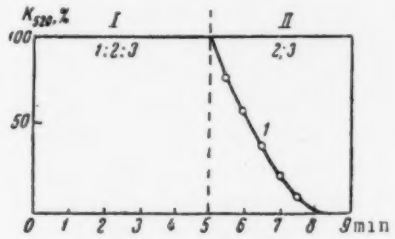


FIG. 1. Photosensitizing effect of chlorophyll a + b absorbate present on surface of adsorbent ($Al_2O_3$) on reduction of methyl red by ascorbic acid. (I) Darkness; (II) Light. (1) Adsorbate; (2) Adsorbate without ascorbic acid; (3) Suspension of $Al_2O_3$ without chlorophyll and with ascorbic acid.

A similar picture was obtained for the chlorophyll adsorbates using a number of other powder-like adsorbents of very different nature. In particular, greater or lesser decolorization of methyl red on illumination by the light absorbed only by chlorophyll in the presence in the solution of ascorbic acid was observed for the pigment adsorbates using calcium phosphate, aluminium hydroxide, ferric oxide, diphenyl silica gel, palmitic acid, barium sulphate, β-carotene, slica, lycopodine, etc. as adsorbents. The rate of the sensitized reaction differed appreciably for the various adsorbents, possibly the chief reason for this was the degree of pulverization and consequently the different surface adsorption. Figure 2 gives the results of experiments with chlorophyll adsorbed on carotene prepared by adsorption from alcoholic solution of chlorophyll.

Since, it was noted that some adsorbents are able to adsorb not only the pigment from the alcohol but also methyl red from aqueous solution, the question arose as to whether the ability we detected for the adsorbed chlorophyll to photosensitize the redox reaction between the ascorbic acid and methyl red was merely an apparent one and that in fact the decolorization of the latter on illumination was due to its adsorption

initated by light, on the powdered adsorbent. However, this was not supported by the results of the following experiments using an adsorbate of chlorophyll on $Al_2O_3$.

(1) The absorption spectrum of the suspended adsorbate in water containing ascorbic acid was measured and then the absorption spectrum of the same adsorbate after the experiment with sensitized reduction of the methyl red. These spectra in the relevant wavelength interval and, in particular, in the region of methyl red absorption were completely identical.

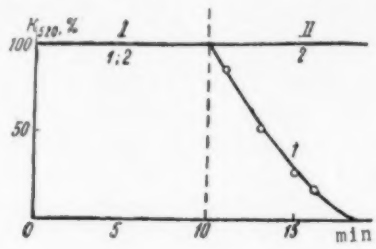


FIG. 2. Photosensitizing effect of chlorophyll $a+b$ adsorbate on powdered β — carotene (particle size 4–10 μ. (I) Darkness; (II) Light. (1) Adsorbate; (2) Suspension of carotene without chlorophyll.

(2) A comparison was made of the absorption spectrum of a solution obtained by extraction the chlorophyll from the $Al_2O_3$ adsorbate with alcohol prior to the experiment with the solution obtained after complete photochemical decolorization of the methyl red. No fundamental difference was noted between these spectra — as should have been the case had methyl red been adsorbed together with the chlorophyll on the $Al_2O_3$.

Evidence that decolorization of the methyl red on illumination was in fact the result of a photochemical reaction is also provided by the results of the experiments using silicic acid anhydride as adsorbent, this strongly adsorbs methyl red from solution on addition to the latter of the chlorophyll adsorbate. The colour of the adsorbate changed from green to red and the green colour only returned after illumination for a time (3–5 min) sufficient for complete sensitized reduction of the methyl red. In the controls without ascorbic acid or with methyl red adsorbate without chlorophyll decolorization did not occur. Hence, even if partial adsorption of the dye on the adsorbent is present, this does not change the situation; the adsorbed dye is also reduced on illumination.

## INFLUENCE OF VARIOUS FACTORS

The rate of the photoreduction reaction of methyl red sensitized with chlorophyll adsorbates is dependent on the amount of chlorophyll on the surface of the adsorbent. Adsorbates on $Al_2O_3$ obtained by adsorption of chlorophyll (see above) from alcoholic solutions of diminishing concentrations showed a corresponding decrease in the rate of the sensitized reaction. Figure 3 (A) gives the results of several experiments. These findings unequivocally point to the direct rôle of the pigment in the process of sensitized photo-reduction.

The rate of the sensitized reaction bore a direct relation to the amount of the adsorbate suspension in the solution. The rate increased with increase in the concentration of the suspension to a limit, naturally, after which the screening effect of the adsorbent started to make itself felt.

As was to be expected, the rate of the sensitized reaction was directly related to the intensity of illumination, as illustrated by Fig. 3 (B). The sensitized reduction was slowed down on decreasing the intensity of illumination.

The temperature of the experiment also exerted a marked effect on the rate of decolorization of methyl red. As can be seen from Fig. 4, the reaction was clearly retarded on lowering the temperature from 40 to 0°. Owing to the complexity of the system it is difficult to decide without ambiguity the causes of such a temperature effect. Besides the possibility that it is a matter of the usual effect of temperature on the stages of the process of proton transfer in the dark, it may be assumed that in this case temperature exerted an effect on the rate of the primary photochemical formation of the labile photoreduced form of the pigment since on changing the temperature, the mobility of the molecules of the proton donor is changed and, finally, temperature may have an effect on the state of the pigment in the adsorbate.

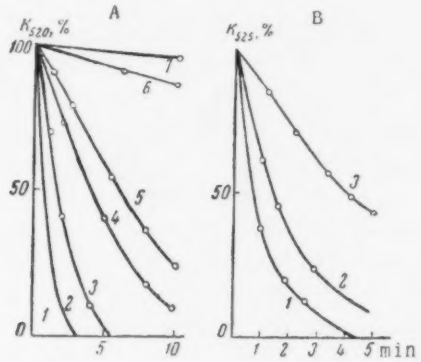


FIG. 3 (A). Effect of amount of chlorophyll present on surface of adsorbent ($Al_2O_3$) on rate of sensitized reduction of methyl red by ascorbic acid. (1–7) adsorbates obtained from differing concentrations of alcoholic chlorophyll solutions. $K_{665}$ of chlorophyll solution equal to (1) 336; (2) 168; (3) 84; (4) 17; (5) 8; (6) 2; (7) control without chlorophyll. (B) Effect of intensity of illumination on rate of reduction of methyl red by ascorbic acid sensitized with chlorophyll $a+b$ adsorbate on $Al_2O_3$. Abscissa, time of illumination; 30 V, 400 W incandescent lamp with voltage; (1) 30 V; (2) 20 V; (3) 15 V.

In investigations of the sensitizing powers of suspensions of aggregated chlorophyll in water [8, 9] we noted that the difference in the rate of the reaction of sensitized photo-reduction of the methyl red in anaerobic and aerobic conditions was incomparably less than for solutions in organic solvents. While in the latter the sensitized reaction in the presence of oxygen did not proceed despite a considerable concentration of ascorbic acid and required a certain induction period (removal of oxygen from solution through sensitized oxidation of ascorbic acid) in order for the reaction to start, in

the heterogenous system in water there was almost no difference in the rate of the reaction in the presence or absence of oxygen. A similar picture was observed in the case of the chlorophyll adsorbates so that a large number of the experiments described were done without evacuation of the air.

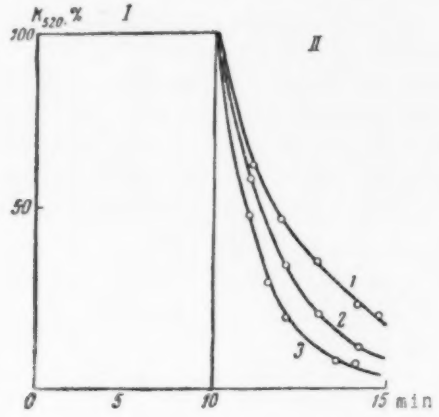


FIG. 4. Effect of temperature on rate of reduction by assorbic acid of methyl red photosensitized with chlorophyll $a+b$ adsorbate on $Al_2O_3$. (I) Darkness; (II) Light. (1) 0°; (2) 20°; (3) 40°.

We have argued [9] that this can to a certain extent be explained by the considerably lower solubility of oxygen in water than in organic solvents. For example, while at room temperature and a pressure of about 760 mm, the concentration of dissolved oxygen in the organic solvents is about $7–20\times10^{-3}$ mole/l., for water it is at most equal to $1\times10^{-4}$ mole/l., i.e. 1·5–2 orders of magnitude less [15]. It is natural that such a small amount of oxygen is removed from solution in the first moments of illumination and that the anaerobic conditions created promote the course of the sensitized reduction of the acceptor. In order to discover whether the explanation indicated above of the minor difference in the rates of the sensitized reaction in heterogenous conditions is actually a matter of the lower solubility of oxygen in water, we carried out experiments with photosensitization of reduction of methyl red by ascorbic acid using haematoporphyrin which is soluble in water. It was found in this case that there was some acceleration in the reaction following the removal of oxygen. Apparently, a definite rôle is played in the phenomenon under study by the specificity of the mechanism of sensitization on the surface of the particles of the pigment or the adsorbate. The rate of the sensitized reduction of methyl red is closely dependent on the reaction in the medium. As in the case of a suspension of chlorophyll [9] replacement of the ascorbic acid by sodium ascorbate or phenylhydrazine hydrochloride had a very obvious retarding effect on the photodecolorization of the dye.

We carried out the reduction of methyl red photosensitized by chlorophyll adsorbed on $Al_2O_3$ in a medium of $D_2O$ instead of $H_2O$ and found no retardation but rather

acceleration of the rate of the reaction. This is at first sight at variance with the findings on the temperature effect on the rate of the reaction. It might have appeared that with proton transfer in the dark, the reaction should have been slowed down by replacing $H_2O$ by $D_2O$. However, the complexity of the system precludes any well-defined conclusion on temperature or $D_2O$ effect. As was stated above, it is possible that the temperature effect is conditional on stages in the process not directly connected with proton or electron transfer.

## ABSORPTION SPECTRA OF CHLOROPHYLL ADSORBED ON VARIOUS MATERIALS

Measurement of the absorption spectra of the adsorbed chlorophyll presented certain difficulties since the large scatter of light over the entire spectrum by the suspensions results in the absorption curves being very flat with maxima which are not easily distinguished. However, our task was not to obtain precise absorption spectra over the entire visible spectrum and we confined ourselves to determining the position of the maximum in the red, which though far from being anything like as sharp as for solutions was quite distinct enough to enable its position to be determined with an accuracy of $\pm 2$ mμ, using a Beckman or SF-4 spectrophotometer. To reduce the sedimentation of the particles of the adsorbate during measurements, we increased the viscosity of the medium by adding considerable amounts of glycerol.

VOL. 5 1960

TABLE 1. POSITION OF ABSORPTION MAXIMUM IN RED FOR CHLOROPHYLL $a+b$ ADSORBATES ON VARIOUS ADSORBENTS

| Adsorbent | Solvent from which adsorption effected | Position of red maximum (mμ) | Adsorbent | Solvent from which adsorption effected | Position of red maximum (mμ) |
|---|---|---|---|---|---|
| ZnO | Ethanol | 665 | $Al_2O_3$ | Ethyl ether | 670 |
| $Ca_3(PO_4)_2$ | ,, | 670 | ,, | Dioxane | 680 |
| Haemoglobin | ,, | 665 | ,, | Acetone | 670 |
| Silica | ,, | 665 | ,, | Toluene | 670 |
| Globulin | ,, | 665 | ,, | Methanol | 670 |
| Hair keratin | ,, | 665 | ,, | Ethanol | 665 |
| $Al_2O_3$ | ,, | 670 | ,, | ,, | 665 |

The findings in the, table show that for our method of preparing the adsorbates, the shift in the position of the absorption maximum in the red for adsorbed chlorophyll in relation to its position for solutions (660–665 mμ) was small and did not as a rule exceed 5–10 mμ. It may thus be concluded that the layers of adsorbed pigment do not have the high degree of orderly aggregation for which a considerable shift in the maximum to the longwave side is characteristic [2].

The degree of aggregation of the adsorbed chlorophyll was related to the nature of the solvent from which adsorption was effected. Dioxane which gives a higher degree of aggregation in films obtained by evaporation of pigment solutions in this solvent [16] also differed in the case of the adsorbates. The shift in the spectrum using dioxane solutions was the greatest.

It should be noted that the special experiments to compare the photosensitizing ability of the chlorophyll adsorbates on $Al_2O_3$ obtained by adsorption from different solutions demonstrated no appreciable differences for the various solvents. In particular, in identical experimental conditions, the rate of decolorization of methyl red was uniform for the adsorbates of chlorophyll obtained from ethanol and dioxane.

## SENSITIZING EFFECT OF ADSORBATES OF PIGMENTS ALLIED TO CHLOROPHYLL

The adsorbates prepared by adsorbing the pigments on $Al_2O_3$ from solutions of protochlorophyll, bacteriochlorophyll and pheophytin also displayed a distinct ability to photosensitize reduction of methyl red by ascorbic acid in aqueous medium. Figure 5 gives the results of the relevant experiments. It should be noted that the slope of the decolorization curves for the individual pigments in Fig. 5 does not given an indication of the relative efficiency of the sensitization by the various pigments since the concentration of the solutions from which the adsorbate was derived was not taken into account.

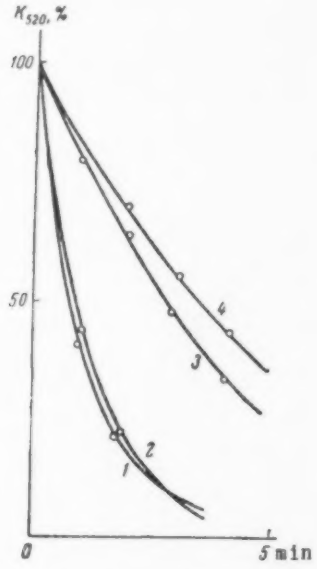


FIG. 5. Sensitizing effect of adsorbates of bacteriochlorophyll, protochlorophyll and pheophytin on reduction of methyl red by ascorbic acid. Abscissa, time of illumination. (1) Chlorophyll; (2) Pheophytin; (3) Protochlorophyll; (4) Bacteriochlorophyll.

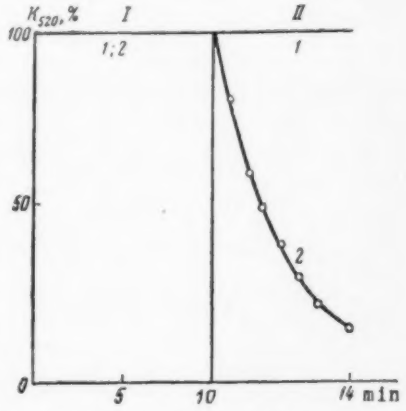


FIG. 6. Sensitizing effect of adsorbates of Cu-pheophytin on reduction of methyl red by ascorbic acid and absence of such effect in case of solution of this pigment. (I) Darkness; (II) Light. (1) Solution in ethanol or pyridine; (2) Adsorbate on $Al_2O_3$.

Interesting results were obtained using a copper complex of pheophytin obtained by replacing the atoms of hydrogen in the centre of the molecule of pheophytin by a copper atom [17]. In organic solvents this pigment does not display photoreducing

ability with either ascorbic acid or phenylhydrazine; this is apparently due to the "quenching" effect of the paramagnetic copper atom on the metastable excited state of the porphyrin molecule [10].

We investigated the ability of the copper complex of pheophytin to sensitize redox reactions in solutions. It was found that in pyridine and ethanol solutions this pigment does not exert photosensitizing action on the reduction of methyl red by ascorbic acid. Negative results were also obtained for a suspension of copper pheophytin prepared by precipitaing its coloidal solution in water [8, 9]. At the same time, the adsorbate of this compound on $Al_2O_3$ as for chlorophyll displayed clear sensitizing ability. Figure 6 shows the results of one of these experiments which indicates that it is possible that in a number of cases adsorption exerts a specific effect on the properties of the pigment molecule. It is possible that in this case binding with $Al_2O_3$ occurred through the additional valency of the copper atom. The latter is, as it were, excluded from the system of the quaternary pyrrole ring of the porphyrin and ceases to exert a "quenching" effect on the metastable, excited state of the pigment molecule. The likelihood of such an explanation is reinforced by the fact that not all the adsorbents activated the copper-pheophytin. For example, for the adsorbates of this compound on ZnO for which the mechanism of adsorption may differ, the ability of copper pheophytin to photosensitize was not detected. The problem of the effect of the adsorbents on the sensitization requires further study.

VOL. 5 1960

## DISCUSSION

The experimental material presented shows that chlorophyll and its analogues in the adsorbed state possess the ability to photosensitize redox reactions. Although in the case investigated by us of reduction of methyl red by ascorbic acid the rôle of the light consisted apparently solely in overcoming the activation barriers and the end products were hardly richer in energy than the original ones, the reaction was accelerated to quite a significant extent by the action of light absorbed by the chlorophyll. This reaction is a convenient one for study, first and foremost because it is irreversible and excludes the difficulties which usually arise in observations of rapidly reversible reactions. In particular, in our experimental conditions we failed to observe reduction of safranin T by ascorbic acid sensitized by the pigment adsorbates, i.e. in a reaction in which the energy of the light is stored in the end products but which is however rapidly and spontaneously reversible in the dark [18].

The mechanism of the sensitizing effect of the adsorbed pigment is still not clear. However, in our view the results suggest that the active rôle here, as in the case of suspensions, is played by the individual molecule of the pigment and it is not essential to have an orderly aggregate of molecules, a requirement for the semiconductor hypothesis of sensitization by chlorophyll. It is difficult to imagine that uniformly aggregated layers of pigment are formed for all the differing adsorbents used by us. The slight shift in the absorption maxima for most adsorbents would rather indicate that orderly aggregation of the molecules is absent or present only to a minor degree.

According to the findings available [2] crystalline chlorophyll has a maximum in the red shifted to the region of 720–740 mμ.

Manifestation of the sensitizing effect apparently requires only successive contact of the pigment molecule in the long-lived excited state and the electron donor and acceptor. In the case of suspensions of aggregated chlorophyll [8, 9], the active molecules are those present at the surface of the particles of the suspension, in the case of adsorbents the adsorbed molecules not enclosed by other molecules. Support for the special rôle of the surface molecules is also offered by the fact that the absolute amount of chlorophyll necessary for the manifestation of a given intensity of sensitization (rate of reaction) in the case of chlorophyll adsorbates is appreciably less than for suspensions of chlorophyll where a considerable number of pigment molecules are contained within a particle.

It is quite probable that the presence of the adsorbent facilitates contact between the molecules of the sensitizer and the reacting molecules. In particular, it is possible that the large molecules of the dye-acceptor, even if in small number, lie on the adsorbent next to the pigment molecules and apparently form a complex with them. This assumption is supported by the experiment with silica anhydride which strongly adsorbs both pigment and dye and which on illumination with red light gives complete decolorization of the dye. If we were to consider that the dye and pigment were separated from each other on the adsorbent, then it would be difficult to understand how sensitization of the reduction of the dye could occur on illumination with light absorbed only by the pigment.

The sensitized reduction proceeds apparently via formation of a very short-lived reduced form of the pigment, of the type of the free ion radical of the semi-quinone of the pigment formed by acceptance by the excited metastable pigment molecule of an electron from the donor. With the presence in the solution of an acceptor, transfer to it of the electron occurs on contact between its molecule and the reduced ion radical of the pigment fixed on the surface, whereas in the presence of a complex with the acceptor this takes place directly within the complex.

## SUMMARY

It has been shown that the reaction of photoreduction of methyl red by ascorbic acid in aqueous solution is sensitized by chlorophyll adsorbed on adsorbents of various types. The rate of the reaction is dependent on the nature of the adsorbent, the concentration of the adsorbed chlorophyll, the intensity of illumination and temperature.

Adsorbates of pheophytin, protochlorophyll and bacteriochlorophyll are also capable of such sensitization.

Measurements of the absorption spectra of the adsorbates showed the presence of relatively minor shifts in the absorption maxima as compared with solutions.

Some ideas are given on the mechanism of the photosensitizing effect of the pigments in the adsorbed state.

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* There is a translation into English of this work.


VOL. 5 1960

# A POSSIBLE MECHANISM OF THE ELECTRICAL ASYMMETRY OF TISSUES*

L. A. MILIUTIN

Urals Gorki State Institute, Sverdlovsk

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MANY organic compounds possess electron conductivity commensurate with the conductivity of electron semiconductors. The excitability and electrical conductivity of living tissues display characteristics peculiar to semiconductor instruments [1, 2]. Excitation is regarded as a certain electronic process, accompanied by liberation of electrons [2]. On the basis of extensive experimental material it is now generally recogniz-

* Biofizika **5:** No. 5, 609–611, 1960.

ed in physiology that biopotentials are an expression of tissue excitation. The locally damaged muscle or nerve possesses electrical asymmetry. Since the time of Vvedenskii [3] the constant injury e.m.f. has been regarded as a manifestation of steady non-oscillatory excitation-parabiosis.

Asymmetry of the electrical state is also noted between the tendon and muscle areas of conical muscles not in a state of excitation or local damage. It is natural to seek an electron-semiconductor basis for electrical asymmetry.

From his measurements of the e.m.f. between the blunt and pointed ends of various metallic and non-metallic bodies, by means of copper sulphate non-polarizable electrodes, Iakimchuk [4] expressed the view that the appearance of this e.m.f. "is based on a single physicochemical process, necessarily involving a first class conductor and that consequently, animal and plant tissues must possess electron conductivity even if it be only weakly marked" [4]. Iakimchuk supports the statement that a first class conductor is involved in the origin of these "geometrical potentials" by the argument "that in an uncharged cone-shaped first class conductor, the free electrons are likewise unequally distributed and that their content is higher in the region of the apex than around the base".

The electrical asymmetry observed by Iakimchuk can be given a simple explanation without resort to the assumption of unequal distribution of electrons in the uncharged metallic cone and without drawing an analogy between conductivity in the metal and non-metallic body. Without going beyond the postulates of the molecular and ionic theory from which Chagovets [5] proceeded in his analysis of the mechanism of "accumulation currents" it is possible to explain the appearance of an e.m.f. on contact of liquid non-polarizable electrodes with blunt and pointed areas of the surface of a solid body irrespective of the character of its conductivity. On any body completely or partially immersed in aqueous solution of an electrolyte, anions are adsorbed owing to their lower energy of hydration [6]. Next to the layer of negative ions adsorbed on the solid body there is, located deeper in the electrolyte, the diffuse ionic layer of Gouy in which the number of positive ions rises to a certain limit distal to the surface of the solid body. As a whole, an ionic double electrical layer is formed with the negative layer on the surface of the wetted solid phase. The stability of this double layer is greater in the areas with the least slope of the surface of the solid phase than in the areas with the greatest slope (i.e. at the apices). At the apex the external (positive) half of the Gouy layer is readily moved under the influence of various factors. When a liquid electrode is superimposed on the apex the positively charged layer of liquid shifts to the region of relatively lower slope at the periphery of the apex where the stability of the double layer is greater. The result is that the double layer at the apex will be compensated by the cations from the deeper layers of the liquid electrode. Under the electrodes in the region with low slope a strong compensated double layer will appear. The small negative potential formed because of the shift to the periphery of the apex of the screening positive ions causes a potential difference between the electrodes. On the basis of these notions of the instability of the screening layer at the apex of bodies immersed in solutions it is possible to explain the intensified growth

of crystals in the corners and apices and also the intensified electrochemical precipitation of copper from solutions of salts at the apices of iron bodies. Additional passage of current in this case will produce a synergic force in the form of a rise in the density of current at the apices.

In our experiments on the electrical conductivity of muscles and nerves silver chloride and zinc sulphate non-polarizable electrodes were used. The electrodes always occupied definite positions in the muscle and tendon. The direction of the e.m.f. supplied was changed by a switch. The voltage delivered was selected by a potentiometer. The current, depending on the order of magnitude, was measured by a milliamperemeter, microammeter or a mirror galvanometer. The time of the action of the external voltage was recorded by means of a stop-watch graduated in seconds. The test preparation during measurement was kept in a humid chamber.

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960

TABLE 1. VARIATION OF ASYMMETRY OF ELECTRICAL CONDUCTIVITY OF THE SYSTEM "SURAL MUSCLE — ACHILLES TENDON OF FROG" WITH APPLIED E.M.F.

| E.m.f (V) | No of measurements | Current sustained for 1 min (A) | |
|---|---|---|---|
| | | Cathode on muscle | Anode on muscle |
| $4\times10^{1}$ | 20 | $0{\cdot}05\times10^{-3}\pm0{\cdot}005$ | $1\times10^{-3}\pm0{\cdot}005$ |
| $2\times10$ | 30 | $1{\cdot}7\times10^{-4}\pm0{\cdot}05$ | $1{\cdot}9\times10^{-4}\pm0{\cdot}05$ |
| $5\times10^{-2}$ | 20 | $5\times10^{-9}\pm1{\cdot}0$ | $5\times10^{-9}\pm1{\cdot}0$ |

On placing one electrode on the muscle and the second on the tendon or the nerve, asymmetry of electrical conductivity was observed. The asymmetry developed to the full value in 1–2 min and was greater the greater the external voltage applied. The findings for muscle are given in the Table.

Completely identical asymmetry was observed on a model of the nerve-muscle preparation composed of a piece of cottonwool and thread, or a piece of cottonwool and a thread spun from the same piece, and impregnated with saline. In the case of impregnation of the model with double distilled water, no asymmetry was observed.

In all cases the greatest electrical conductivity was observed if the cathode was located at the tendon, the nerve or at the thread. Asymmetry set in slowly and its dependence on sufficient concentration of ions strongly suggests the ionic nature of this asymmetry.

The relatively smaller water content in the thread, the tendon and the nerve (58·3 per cent) than in the porous cottonwool of the model or muscle (75·6 per cent) leads to greater depletion of anions from the dense parts of the system owing to adsorption in the solid phase. The less adsorbable cations were distributed equally in the entire liquid phase of the system. Thus, in the region with a high relative content of solid phase electrical negativity will be observed. By superimposing an external e.m.f. on such a body, asymmetrical in the sense of ion concentration, it is possible to obtain stable high electrical conductivity if the cathode lies on the less hydrated part as the weakly adsorbable cations readily pass by the adsorbing surfaces. For a reverse direction

of the e.m.f. there occurs a rapid fall in electrical conductivity owing to competition for anions on the part of the adsorbing solid phase. Adsorption of anions on the solid phase greatly reduces the effective mobility of these current carriers. Hence, a distinctive "ionic suporting layer" is formed.

Treatment with cyanides and methyline blue exerts an effect on the balance of positive and negative charges in the nerve [7], which may point to the dependence of the "supporting layer" on redox processes.

Without laying claim to a final solution of the problem of electrical asymmetry of muscles and nerves by means of the ionic theory it is necessary in investigating the problem of the rôle of holes or electronic conductivity in living tissues, as systems bearing a relation to electrolytes, to take into account the shunting effect of ionic conductivity.

The "geometric" potential difference and asymmetry of electrical conductivity in the muscle may be explained by adsorption of the anions in the solid phase and by diffusion of the cations in the liquid phase.

VOL
5
196

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* There is a translation into English of this work.

# METHODS AND INSTRUMENTS

## STUDY OF ELECTRICAL CONSTANTS OF RAT LIVER TISSUE*

A. I. POLIVODA and A. A. MIKHAILOVA

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VOL. 5 1960

STUDY of the electrical constants of tissues and cells *in vivo* is of considerable interest from the point of view of their submicroscopical structure. This problem has been repeatedly discussed in the literature

The electrical constants of blood cells and plant cells [1–5], and also muscle tissue [6–9], have received most study. Information has been gained on the electrical characteristics of subcutaneous tissue [10]. A number of findings are available on the electrical conductivity of nerve fibre [11].

The absolute values of the electrical constants of the liver have received less attention. However, the separate *in vivo* measurement of the ohmic and capacitance components of impedance of hepatic tissue according to the frequency spectrum allows certain conclusions to be drawn on the submicroscopic structure of this vitally important organ. In addition, computation of the absolute values of a number of electrical characteristics of the tissue may further contribute to an understanding of the molecular-structural processes developing in the liver in pathological conditions. In the present work measurement was made of both components of impedance in a frequency range from $10^2$–$10^7$ c/s and the absolute values of the electrical constants of rat hepatic tissue were computed.

The electrical conductivity was measured at a temperature of 2–3° in the liver of 30 male rats weighing 150–200 g. The rats were killed immediately before measurement and a specimen of the hepatic tissue weighing 650 mg placed between specially designed electrodes (Fig. 1) the constant of which ($l/S$) was 2·36.

It should be noted that the design of the electrode is of decisive importance for the separate measurement of the impedance components over a wide frequency range, in order words, the electrode must have a wide band.

Wide band electrodes must have the following properties: (a) minimal input (geometrical) capacitance; (b) minimal transfer capacitance; (c) minimal inductance; (d) minimal electrolytic polarization at low frequencies; and (e) possibility of linking up to a co-axial cable with high input resistance.

In addition, because of the considerable "through conductivity" of tissue the geometry of the electrodes must give strictly parallel lines of force and equipotential surfaces. A necessary condition for this is absence of "dead" spaces beyond the electrodes which, in the presence of through conductivity, immediately distort the geometry of the field and lead to the appearance of RC frequency peaks.

The electrodes designed by us (Fig. 1) meet the above-mentioned requirements. An electrical field with parallel lines of force and equipotential surfaces is established within the cylindrical area of the sleeve (1).

The bases of the cylinder are the parallel surface of the electrodes (2) covered with platinum black.

The sleeve is prepared from molybdenum glass, has an aperture for removing air (3) and is fitted with two prominences (4) for the rubber bonds (5).

Platinum electrodes are applied to the polished end surfaces by a special soldering method [12] and are in contact with the platinum leads sealed into the ends (6) fitted with thermal compensators (7). One of the electrodes has a coaxial television joint [8], the other, a bush [9] for earthing.

* Biofizika **5**: No. 5, 612–616, 1960.

Separate measurement of the ohmic resistance (R) and capacitance (C) was made by means of a bridge circuit over a frequency range of $10^2$ to $10^7$ c/s. From the R and C indications the values of the tangent of the loss angle (tan $\delta$) the specific resistance ($\rho$) and the dielectric constant ($\varepsilon$) were computed.

The measuring circuit was in the form of a multirange bridge having four ranges for the ohmic and three for the capacitance components of impedance. The circuit is fitted with detecting stages and a differential amplifier with an adjustable coefficient of amplification of up to $10^4$.

Two generators provide frequency bands from $1\times10$ to $2\times10^5$ c/s and from $1\times10^5$ to $2\times10^7$ c/s with an amplitude of the measuring signal of 0·5–1 V.

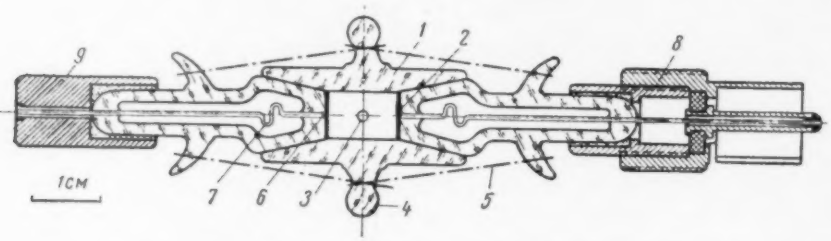


FIG. 1. Electrodes for measuring electrical conductivity of tissue.

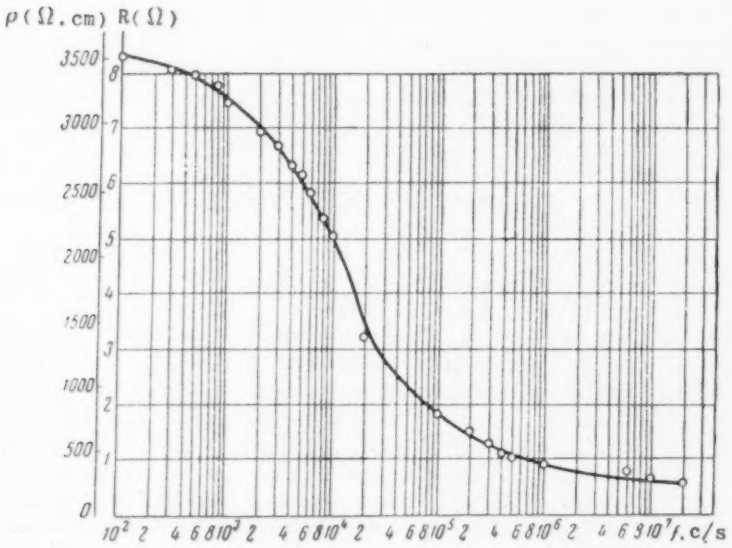


FIG. 2. Specific resistance $\rho$ of rat liver as a function of frequency $F$.

The measurements showed that the ohmic resistance R and the specific resistance $\rho$ of the rat liver have a clearly marked region of dispersion of the order of 80 per cent in the frequency range 1–100 kc/s, changing within limits of 3500–300Ω. cm. The maximum of this dispersion lies at a frequency of 15 kc/s (Fig. 2).

The capacitance C, as also the dielectric constant $\varepsilon$, diminishes with an increase in the frequency over the entire frequency range investigated. While at a frequency of 100 c/s $\varepsilon$ for hepatic tissue is $2{\cdot}8\times10^5$, at 1 Mc/s $\varepsilon$ falls to $2\times10^3$ (Fig. 3).

The tangent of the loss angle (tan δ) drops from a maximum in the region of the lowest frequencies (100 c/s–1 kc/s). In the region of higher frequencies ($5 \times 10^5$ c/s) tan δ displays a second small maximum reaching in absolute terms the value 3·4 (Fig. 4).

The results presented in the graphs were obtained with an accuracy of ±10–15 per cent because of variations in the indications from one animal to another.

As stated, in the literature there is very little information on the absolute values of the electrical characteristics of hepatic tissue and, therefore, the constants measured may be interpreted by comparing them with other tissues and cells.

There are now two points of view on the mechanism of conductivity of living tissues.

The advocates of the membrane theory [1–3, 11, 13] contend that tissue is, in the main, an ionic conductor with numerous partition membranes. All the mechanisms of dispersion of R and ε are attributed by them to accumulation of ions around these boundaries (polarization).

According to the second theory [4–8, 10, 14], together with ionic mechanisms of conductivity there are dipole mechanisms due to bound polar molecules orientated in the electrical field.

VOL. 5 1960

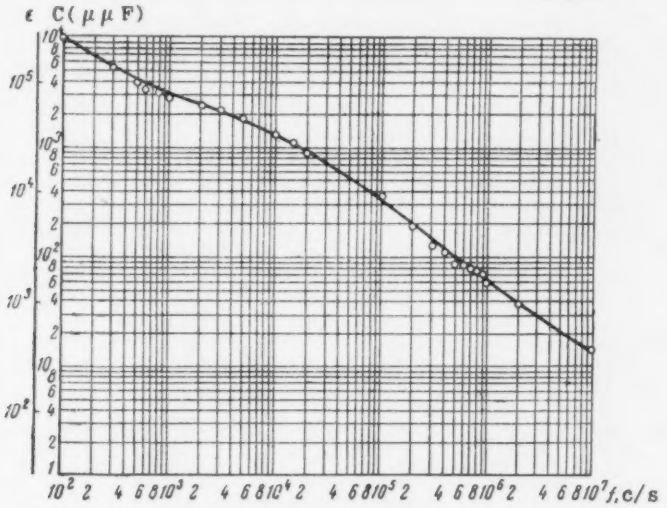


FIG. 3. Capacitance C and dielectric constant ε of rat liver as function of frequency *F*.

Apparently, the conductivity of living tissues at various frequencies must be considered along different lines. Figure 2 shows that the ohmic resistance R and the specific resistance ρ of hepatic tissue display sharp dispersion in the sonic and ultrasonic frequency spectrum —1–100 kc/s). At such low frequencies the resistance of the tissue is undoubtedly affected by the presence of structures (cellular membranes, various phase boundaries in the protoplasm, etc). This is indicated by experiments carried out in plant and bacterial cells [13–15], on erythrocytes [3] and subcutaneous tissue [10]. Therefore it may be assumed that the maximum value of ΔR (on Δρ) at a given frequency characterizes the state of the cellular structures mentioned.

It should be noted that for the liver the value ΔR per unit frequency is very considerable as com pared with other tissues.

Thus, while for the subcutaneous tissue [10] the resistance R at a frequency of 700 kc/s is 2–3 times less than that at a frequency of 1 kc/s, for the liver this ratio is of the order of 8. In addition, as compared with other tissues [10] the maximum ΔR for the liver is sharply marked for a frequency of the order of $10^4$ c/s whereas for the subcutaneous tissue this maximum lies in the frequency region of the order of $10^5$ c/s.

Thus, in the sonic spectrum the liver shows a clearly marked frequency dependance of R, which suggests that it is related in some way to the morphology of hepatic tissue.

At high frequencies of the order of $10^6$ c/s where the phase boundaries no longer can play a considerable rôle in conductivity, the ohmic resistance of the tissue is due apparently, in the main, to the ions of the electrolytes.

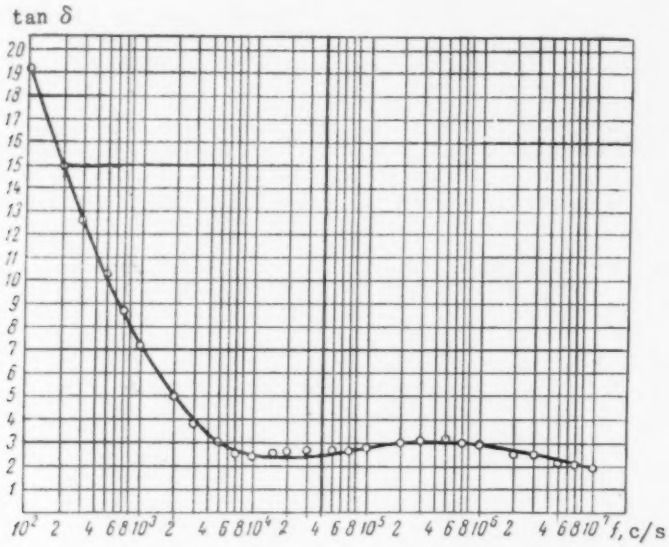


FIG. 4. Tangent of loss angle tan $\delta$ of rat liver as function of frequency $F$.

It is known [16] that in the mineral composition of the liver the main rôle is played by the $Na^+$ ions (60 mg per cent) and $K^+$ (96 mg per cent), constituting 156 mg per cent.

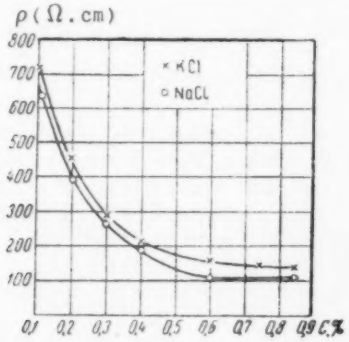


FIG. 5. Specific resistance of electrolyte solutions as a function of concentration $C$.

If it is assumed that the principal part of the conductivity of hepatic tissue at a frequency of the order of $10^6$ c/s is accounted for by these ions, then thier specific resistance $\rho$ in aqueous solution should be equal to 450–480 Ω.cm (Fig. 5). Figure 2 shows that for the liver $\rho$ is quite close to this value (430–450 Ω.cm).

Other indirect evidence for the assumption is the fact that $\rho$ of the electrolyte solution, on elimination of the phenomena of polarization, remains constant up to frequencies of $10^6$–$10^8$ c/s. The specific resistance $\rho$ of hepatic tissue for frequencies greater than $2–4\times10^5$ c/s is no longer fully dependent on frequency (Fig. 2).

Hence, it may be assumed that $\rho$ of hepatic tissue at frequencies of the order of $10^6$ c/s is an indication of the amount of electrolyte in the tissue.

However, these statements are only true as a first approximation. There is no doubt that $\rho$ of hepatic tissue is also accounted for by other ions ($H^+$, $Ca^{++}$, $P^{++}$, etc.) although their amount in the tissues is considerably less as compared with $Na^+$ and $K^+$.

In considering the frequency dependance of the dielectric constant $\varepsilon$ of hepatic tissue it is possible to notice a change in it by several orders of magnitude over the frequency range investigated ($\varepsilon = 8\times10^5$ at a frequency of $2\times10^3$, and $\varepsilon = 5\times10^2$ at a frequency of $5–10\times10^7$ c/s).

Fricke [1] obtained a value of the order of several thousands for $\varepsilon$ in the case of erythrocytes of different concentrations and a frequency of 16 kc/s.

It should be noted that at such comparatively low frequencies the presence of membrane polarization must considerably distort the true value of $\varepsilon$ [17].

Accordingly, we shall not discuss the absolute values of the dielectric constant of hepatic tissue and merely note the overall trend of change in $\varepsilon$ over the frequency spectrum. Figure 3 shows that reduction in $\varepsilon$ of hepatic tissue with an increase in the frequency of the electrical field occurs quite unequally.

According to the theory of Debai [18] inequality of dispersion may point to the presence of relaxation groups and associated electrical losses. This is also indicated by the presence of a maximum tan $\delta$ in the frequency region of the order of $5\times10^5$ c/s (Fig. 4).

Thus, the hepatic tissue apparently contains relaxation groupings with time of relaxation $\tau$ equal to $3\cdot12\times10^{-5}$ sec.

Since the characteristics set out for the rat liver are sufficiently stable they must be considered as structural characteristics of the tissue.

Therefore, it would be of interest to investigate the above enumerated constants when the animal is acted upon by external damaging factors, for example, ionizing radiations. These data may also help to make clear certain aspects of the problem of the *in vivo* structure of tissue.

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# METHOD OF PHOTOELECTRIC COMPENSATION*

M. I. MEKSHENKOV and A. P. ANDREITSEV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
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196

THE phenomenon of luminescence of complex molecules and the corresponding luminescence spectroscopic methods of investigation are widely applied in the solution of a number of important problems of modern biophysics and radiobiology. However, sufficiently elaborate experimental treatment of these problems is at the present time handicapped by the absence of a simple spectral apparatus with objective and rapid recording, this is particularly important in work with biological specimens. A number of the problems of photoelectric recording with complex apparatus are either not solved at all or in an unsatisfactory manner. One way of solving these problems is indicated below, a simple method of automatic photoelectric recording which can be used in general for various optical measurements is described.

The method proposed makes it possible to compensate the variation in intensity of various radiation sources and the supply voltage of the photoelectric receivers and may be used as a corrector in spectrophotometry and also in a number of problems of spectroscopy and pyrometry with comparison of two signals. The method is based on obtaining two electrical signals from the same source which are transmitted to a circuit which makes it possible to obtain a ratio of these two signals at the output.

In principle, nearly all the problems enumerated can be solved with the optical arrangement shown in Fig. 1. The light from a gas discharge or any other source S used in the spectrophotometer is collected by the condensor C in the plane of the slit of diaphragm D (depending on the aim of the work this may be the entry or exit slit of the spectral instrument, see below).

Part of the flux from mirror M is directed to the auxiliary photocell $P_2$, the main flux falls on the test object O, after which the light converted in the object falls on the recording photocell $P_1$.

It is clear that the photocurrent of each of the photocells is proportional to the strength of the flux $E_l$ obtained from the lamp, i.e. $I_{p1} = k_1 E_l$ and $I_{p2} = k_2 E_l$, where $k_1$ and $k_2$ are constants independent of the value $E_l$. If now, by some means or other, a signal is obtained equal to the ratio of these photocurrents, i.e. $I = I_{p1}/I_{p2} = k_1 E_l/k_2 E_l$, then this signal will not depend on the intensity of the radiation source. Hence, compensation of the variations in intensity is achieved. Further, if both photodetectors (photomultipliers) are supplied from the same voltage source (see Fig. 2 (a)) then we can write $I_{p1} = k'_1 f(U_n)$, $I_{p2} = k'_2 f(U_n)$.

Then the output signal $I = I_{p1}/I_{p2} = k'_1/k'_2$ does not depend on the voltage supply $U_n$. Thus, compensation for the variations in the supply voltage of the photoelectric receiver is achieved. If only stabilization of the voltage $U_n$ is required, without consideration of the variation in intensity of the

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light source, this can be achieved by use of one photoelectric receiver with the scheme in Fig. 2 (b) (see also curve in Fig. 5).

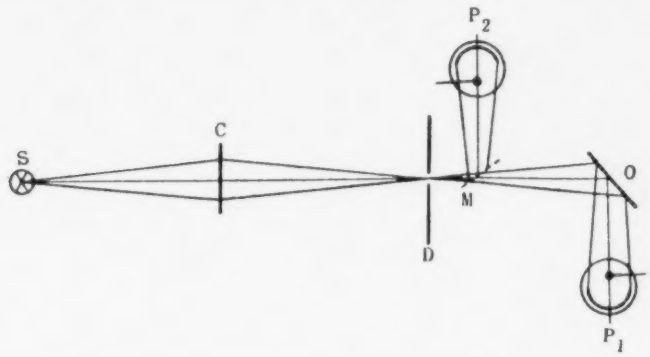


FIG. 1. Optical arrangement for photoelectric compensation. (S) Radiation source; (C) Condensor; (D) Diaphragm (slit); (M) Mirror; ($P_1$) Recording photocell; ($P_2$) Auxiliary photocell; (O) Object.

In spectrophotometry, correction for the selectivity of the spectral sensitivity of the photoelectric receiver $k_{\lambda P}$, the optical reflexion factor $k_{\lambda O}$ and the unequal distribution of energy in the spectrum of the radiation source $k_{\lambda U}$ is achieved in the manner already described, by placing the photocells $P_1$ and $P_2$ behind the exit slit of the spectral instrument. It is clear that the coefficients $k_1$ and $k_2$ may be represented in the form;

$$k_1 = k_{\lambda U} \times k_{\lambda P} \times k_{\lambda O} \times k''_1, \quad k_2 \times k_{\lambda U} \times k_{\lambda P} \times k_{\lambda O} \times k''_2$$

Thus, $I = k''_1/k''_2$ does not depend on the factors enumerated above. It is self-evident that the coefficient $k_1$ in all the cases considered depends on the characteristic of the test object being measured $k_{\lambda Ob}$, i.e. $k_1 = k''_1 \times k_{\lambda Ob}$, so that in all cases the resultant signal $I = k_1/k_2 = (k''_1/k_2)\, k_{\lambda Ob}$ will depend on this value which may represent the spectral coefficient of transmission, reflexion or absorption,

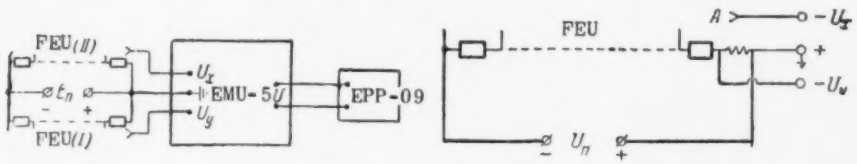


FIG. 2. (a) Block circuit diagram for electronic compensation; (b) circuit for recording signals for compensation of variations in voltage of FEU supply sources.

the spectral or integral energy yield of luminescence. Finally, on the basis of the method described a so-called red/blue pyrometer can be constructed. The optical scheme of such a pyrometer is fully analogous to that in Fig. 1 where the source S is replaced by an incandescent body the temperature of which is to be measured. In front of the photocells $P_1$ and $P_2$ red and blue interference filters are mounted respectively.

We tested and applied in a recording spectrophotometer devized by the authors, two types of circuits performing the operation of division. The first is based on the compensation method of voltage measurement used in electronic potentiometers.

The basic circuit diagram for the introduction of the signals into the measuring circuit of the potentiometer is shown in Fig. 3. As the Figure shows the basic circuit of division is identical with the EPP measuring circuit where the supply of the bridge (arm CD) has been replaced by the voltage from the auxiliary photocell $P_2$. The cathode follower CF while not playing a fundamental rôle makes it possible for the allowed photocurrents to produce the necessary supply voltage for the bridge ($\sim$ 1·5 V). It is easy to show that this circuit actually makes it possible to obtain deviations in the slide wire of the potentiometer with an accuracy proportional to the ratio of the signals $U_x$ and $U_y$ from the photocells $P_1$ and $P_2$ respectively. Kirchoff's law for the individual currents at the moment of equilibrium

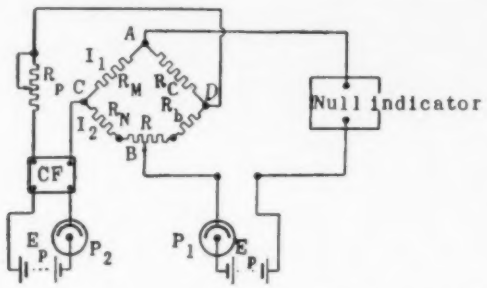


FIG. 3. Basic circuit diagram for electro-mechanical division. $E_p$ denotes supply of FEU from two separate symmetrical selenium rectifier bridges which are fed from two identical secondary windings of the same step-up transformer.

of the circuit gives:

$$\begin{aligned} U_y &= I_1(R_M+R_C)+R_p(I_1+I_2) \\ U_y &= I_2(R_N+R+R_b)+R_p(I_1+I_2) \\ U_x &- R_C\times I_1+I_2(R_bR_x)=0 \end{aligned} \tag{1}$$

Here, $R_x$ is the component of $R$, corresponding to the position of the slide $B$.

Since $I_1+I_2=I$ and $I=q\times U_x$, where $q=R_p/R_e$ with $R_e$, the equivalent resistance of the bridge including resistance $R_p$ we get:

$$U_y(1-q)=I_1(R_M+R_C) \tag{2}$$

$$U_y(1-q)=I_2(R_N+R+R_b)$$

$$I_1=U_y(1-q)/R_M+R_C,\ I_2=U_y(1-q)/R_N+R+R_b) \tag{3}$$

Substituting (3) in the third equation in (1), we obtain:

$$U_x=R_C\frac{U_y(1-q)}{R_M+R_C}-(R_b+R_x)\frac{U_y(1-q)}{R_N+R+R_b} \tag{4}$$

or, introducing the symbols $A$, $B$ and $C$ for the coefficients depending on the constant resistances of the bridge, we get:

$$U_x=AU_y-BU_y-R_xCU_y \tag{5}$$

whence

$$R_x=\frac{1}{C}\frac{U_x}{U_y}+\frac{A}{C}-\frac{B}{C}$$

Hence, the position of the slide wire B, which corresponds to a definite $R_x$, is determined by the ratio of the signals from $P_1$ and $P_2$.

Together with the electromechanical method described for obtaining the ratio, an electronic circuit for division was tested. For this purpose we used the multiplier-division arrangement of the EMU-5 type of the Institute of Automation and Telemechanics of the U.S.S.R. Academy of Sciences [1] with which it is possible to perform the operations of division and multiplication for a wide range of real numbers. The block diagram of the circuit tested is shown in Fig. 2 (a). The results of testing by both methods are given in Fig. 4 in the form of photographs of the curves of heating of a value, recorded on EPP-09 tape, without a compensatory circuit (a) and with compensation (b). It is clear from the photographs that variations in intensity within the limits 25–30 per cent made practically no difference

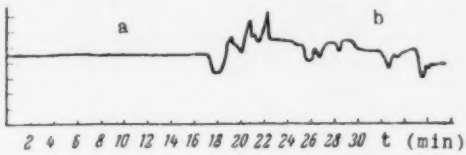


FIG. 4. Record of operations of a value (a) with compensation circuit; (b) without compensation Ordinates, level of output signal (relative units); abscissa, time (min).

to the value recorded. The electronic division circuit gives full compensation for practically 100 per cent changes in the intensity of the light flux of the radiation source. It should be noted that the compensation method proposed for all the applications listed requires only a very small range of change in the voltages supplied to the dividing device (in any case not more than 1–1·5 V) and this range can be selected in the most convenient region of output voltages. It is thus possible to replace comparatively complex electronic dividing instruments approximating the logarithmic curve for a wide region of input voltages by a simpler electronic circuit which can be assembled on the basis of the circuit described in the literature for obtaining antilogarithms [2].

Analysis of the anode-grid characteristics of some values with the variable $\mu$ shows that in the interval of input voltages of the order of 1–1·5 V the logarithmic relation $I_a = \log U$ may be obtained with a quite sufficient degree of accuracy.

In conclusion, we would note the following advantages of the method described.

(1) Up to now in various optical measurements stabilization of the intensity of the radiation sources has been achieved by two different methods. The first consisting of the usual electronic stabilization of the voltage or supply current of the lamp. The shortcomings of this method are that it is

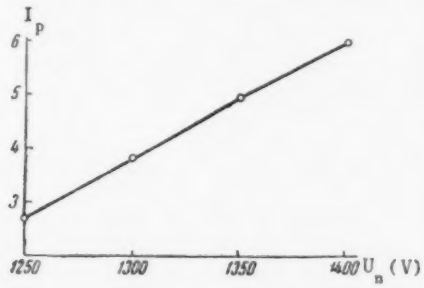


FIG. 5. Relation between photocurrent of FEU and supply voltage $I_p$, photocurrent at output of cathode follower CF (mA).

necessary to have a powerful voltage stabilizer (1·5–2 kW) and that this method does not remove the instability of the light flux caused by the spontaneous displacement with respect to the electrodes of the filament of the superhigh-pressure gas discharge lamp.

The second method proposed by Shirokov [3] is based on application of optical feedback in the amplifier which controls the powerful electronic 6 N5 values connected in parallel to the SVDSh-250-3 mercury lamp. The circuit makes it possible to remove fluctuations both in the constant component and the rapid jumps in intensity. However, there are severe limitations on its applications. The power of the circuit proposed by Shirokov allows stabilization of the light flux within the limits of change in the supply voltage of the lamp of the order of 10 V. Application of Shirokov's method for more powerful radiation sources and for more thorough stabilization would lead to the need for considerable increase in the power of the stabilizing electronic circuit and the rectifier supplying the radiation source. In addition, the method requires that the lamp be supplied with d.c., i.e. needs a powerful rectifier. Another defect is that because of the very essence of the method, its effect is achieved through highly unstable electrical operation of the lamp which naturally affects its length of life. The compensation method proposed here does not fundamentally involve the system of operating the lamp which is supplied from the a.c. mains. In addition, the electronic circuit does not superimpose any restrictions on the "depth" of change in intensity of the surface being illuminated (the planes of the slit of the spectral instrument or the object itself).

(2) The above-described method of stabilization of the supply voltages for the photoelectric devices differs in principle from the existing method of electronic stabilization. In effect, without stabilizing the voltage *per se*, it compensates those fluctuations which develop in the photocurrent. The method is simple and can be widely used — especially with the circuit depicted in Fig. 2 (b). The only requirement imposed by the method on the elements of the circuit — linearity of change of the photocurrent in relation to the supply voltage — is satisfied with sufficient accuracy within the limit $\Delta U_n$ (= +5%) (Fig. 5). In connecting the circuit as in Fig. 2 (a) the limitation on non-linearity and, consequently, on the value of $\Delta U_n$ is largely removed by matching the pair of photomultipliers or the photo-elements for a uniform relation $I_P = f(U_n)$.

(3) Correction of the various spectral curves plotted by means of the spectrophotometers is achieved by the above-described method which unlike the existing ones is purely photoelectrical. It is possible to make universal use of this for sources with any spectral distribution of energy and receivers with any spectral distribution of sensitivity. At the same time, it is known that the mechanical correction applied for example in such spectrometers as the ZMR and DMR and others is suitable only for a given pair of sources and receivers. The same defect mars the method of the optical wedge applied in the spectrophotometer described in [4].

(4) The operation of comparing two signals by obtaining their ratio — necessary in spectrophotometry and photocolorimetry when measuring for example such optical characteristics as the coefficient of absorption or reflexion, the quantum of energy yield of luminescence and also for obtaining the red/blue ratio — is solved here by the automatic photo-electric circuit.

It should be emphasized that the method described solves simultaneously all the problems met by means of the same photoelectric circuit, whereas in existing recording devices each of them is solved by a separate special apparatus.

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# THE DYNAMOCARDIOGRAPH *

YE. B. BABSKII, A. B. VENEDIKTOV, V. L. KARPMAN and M. I. TISHCHENKO

Institute of Normal and Pathological Physiology, U.S.S.R. Academy of Medical Sciences, Moscow; The Design-Technological Bureau of Biophysical Instrumentation, Leningrad

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VOL. 5 1960

THE bases of the technique of dynamocardiography were evolved as early as 1951 and have been described in a number of papers [1–4]. Dynamocardiography makes it possible to record directly the internal displacements of the centre of gravity, due to cardiac activity, of the individual parts of the body and, in particular, the thorax. This technique also allows recording of the internal displacements of the centre of gravity of the whole human body lying on the recording arragnement of the dynamocardiograph. The internal displacements of the centre of gravity (both total and in part) are determined from the displacement of its projection in the plane of the recording device.

The physical bases of dynamocardiography distinguish in principle this technique from ballistocardiography which also serves for the investigation of mechanical manifestations of cardiac activity.

Dynamocardiography makes it possible to determine the direct internal shifts in the centre of gravity whereas ballistocardiography records the reaction of the human body for the same internal shifts in the centre of gravity [5].

The technique of ballistocardiography has a number of fundamental defects which have been repeatedly noted in the literature. The most important are; resonance distortions due to vibrations of the body (high-frequency and direct ballistocardiography), the non-linear dependence of the amplitude of vibrations of the body on the applied force (direct ballistocardiography), the need for damping, causing definite distortions in the curve (ultra-low-frequency and low-frequency ballistocardiography). As a result of these defects, quantitative analysis of force in the ballistocardiograms is extremely difficult. The problem of eradicating all the defects of ballistocardiography has led to the design of various types of instruments. At present, this problem has not yet been solved.

Dynamocardiography is free of the main defects of ballistocardiography. Being an aperiodic, non-resonant instrument, the dynamocardiograph makes it possible without distortions to record the force interrelations developing in the human body from the work of the heart and the movement of the blood in the vessels. Vivid proof of this is provided by the possibility of analysing of the time the individual phases of the cardiac cycle [6] with an accuracy not inferior to the results obtained on intracardiac sounding [7].

Despite a number of positive aspects of dynamocardiography and its clinical promise already demonstrated in a number of papers [8–13], wide application of this technique has been impossible because of the absence of an instrument suitable for operation in conditions of clinical institutes. The instrument described and intended for mass production bridges this gap.

The dynamocardiograph of the DKG-01 type together with a photorecording device is an instrument with which it is possible to carry out graphic recording of the dynamocardiogram (d.c.g.) — the curve of displacement of the centre of gravity of the human thorax in a longitudinal or transverse direction. The principle of action of the instrument is based on the conversion of the moment of the forces acting on the pick-up instrument into electrical signals with their subsequent amplification and photorecording on a paper strip.

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The instrument is intended for recording d.c.g. by means of loop galvanometers of the MOV-2 No. 4 or No. 5 type or other ones of similar parameters. The dynamocardiograph comprises the following units (Fig. 1); (1) the pick-up instrument, (2) the amplifier unit and (3) the supply unit. The units are assembled on mobile table which simplifies operation of the instrument in clinical conditions.

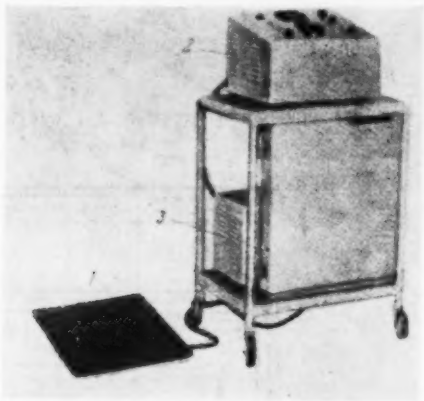


FIG. 1. General view of DKG-01 dynamocardiograph with recording arrangement.
(1) Recording apparatus; (2) Amplifier unit; (3) Supply unit.

The principle of the pick-up instrument is the recording of the changing moment of the forces, on the bases of amplitude modulation of a carrier frequency by means of tenso-sensitive bridges, and has previously been described [1]. However, the design of the pick-up instrument of the DKG-01 fundamentally differs from that published earlier (Fig. 2). The flat recording instrument connected under the thorax of the supine human subject measures $350 \times 350 \times 20$ mm and consists of a base (1) of rectangular shape on which are located eight flat springs fixed at one end (2) (5) and (6) and a cover (4). The springs are the elastic element of the system and have the shape of an elongated trapezium. This shape is designed to meet the requirements of uniform resistance of the spring to bending. Since the load is applied to the free end of the spring working as a cantilever beam, choice of this shape increases the efficiency of the resistance tensometers sealed in the plane of the working springs because of their more uniform deformation. The four springs (2) with their free ends in the corners of the recording instrument are supporting ones and take up all types of load. By means of screws (3) the supporting springs are connected to the cover (4) and thus join the cover to the base. The four other springs are the working ones, i.e. receiving the moments of the force in the longitudinal (5) and transverse (6) directions.

The working springs receive only the vertical displacements of the cover with respect to the base. This is achieved by interposing steel balls (7) between the free ends of the working springs and the cover. Because of the rolling of the cover the balls, twisting of the working springs is excluded on horizontal displacement of the cover. Thus, the springs work only on bending, i.e. complete mechanical separation of the components is achieved. This ensures qualitative recording of the d.c.g. without the use of compensation circuits.

As our investigations showed, in determining the weight of the part of the thorax lying on the pick-up instrument, the tenso-sensitive bridges of which were connected up not in a difference but a sum circuit (tensometrically) the weight taken up by the recording device was about a quarter of the total weight of the body and did not exceed 30 kg even in cases of obesity. Therefore, the weight of 30 kg was chosen as the maximum force acting on the pick-up device. Starting from this condition the following parameters for the springs were selected (number of springs, eight)

1. Material — U8A steel; $E = 2{\cdot}1 \times 10^6$ kg/cm$^2$
2. Length of working part of spring, $l = 80$ mm
3. Width of spring at base, $b = 22$ mm
4. Permissible deflexion of end spring, $f = 1{\cdot}5$ mm
5. Permissible bending stress, $\sigma = 5000$ kg/cm$^2$
6. Thickness of spring, $h = 3$ mm.

As is known, the sag of a cantilever beam is determined from the formula:

$$f = \frac{Pl^3}{2EI} \tag{1}$$

where $P = 30/8 = 3{\cdot}75$ kg; the moment of inertia, $I = bh^3/12$. On substituting the corresponding values in formula (1) we obtain $f = 1{\cdot}3$ mm. With a further increase in the load the free ends of the springs enter into contact with the base since the clearance between the springs and the base is 1·5 mm. Thus, because of the limitation of the deflexion of the springs the springs and tensometers are automatically protected from extreme overloading.

VOL. 5 1960

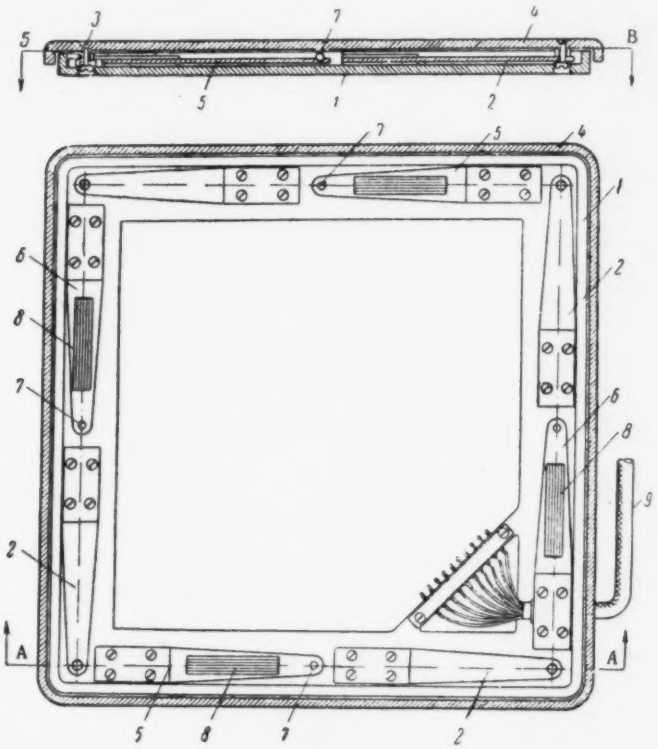


FIG. 2. Diagram of recording arrangement. (1) Base; (2) Supporting springs; (3) Clamping screw; (4) Cover; (5) Working springs for recording longitudinal dynamocardiogram (d.c.g. I); (6) Working springs for recording transverse dynamocardiogram (d.c.g. II); (7) Ball; (8) Ohmic resistance wire; (9) cable.

The two working springs, the balls of which are in the long axis of the cover of the recording device, serve to record the moments of the force applied along the longitudinal axis, i.e. the longitudinal d.c.g.

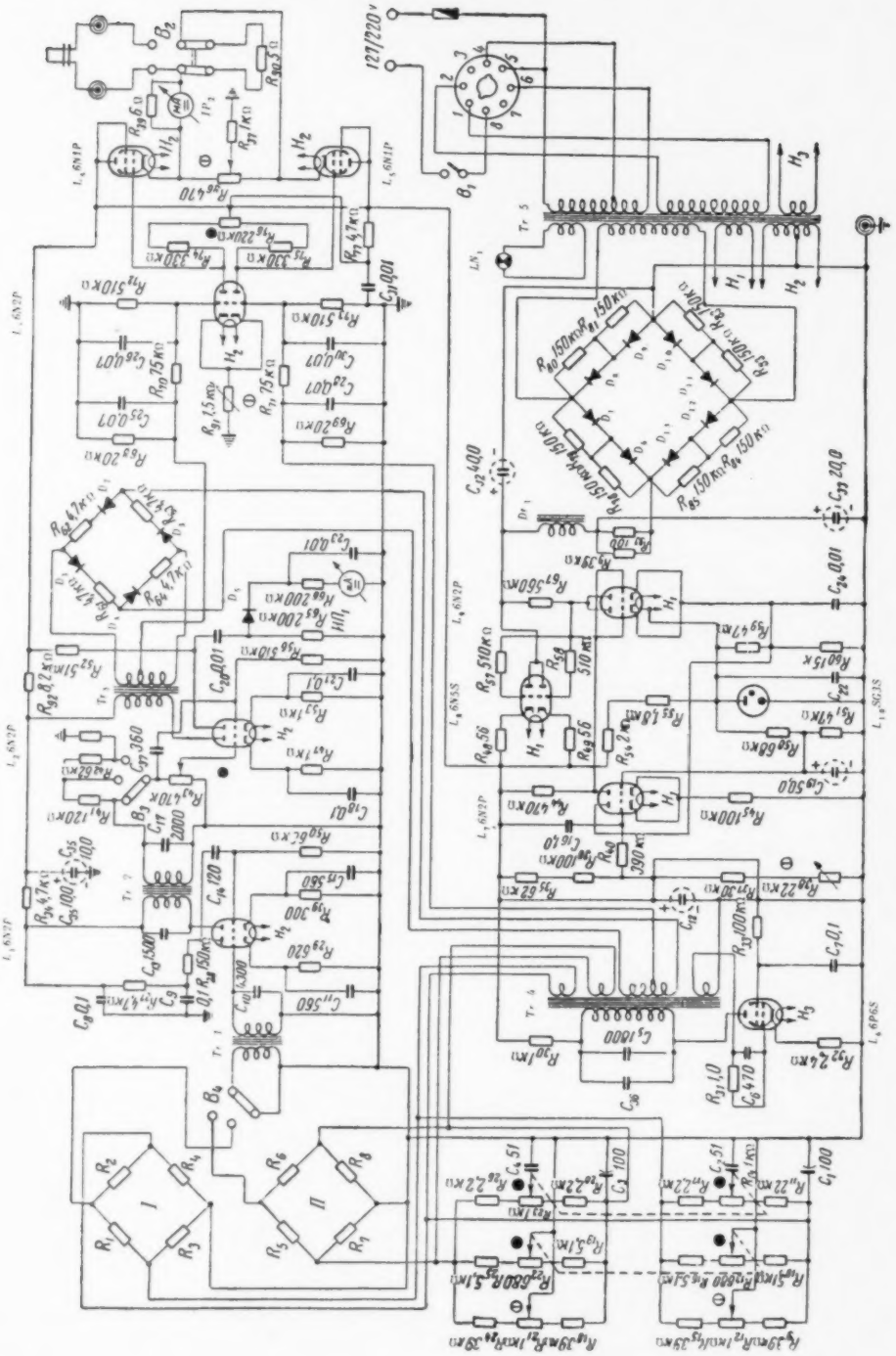

FIG. 3. Basic circuit diagram of dynamocardiograph DKG-01.

(d.c.g. I, bridge I in the basic circuit diagrams — see Fig. 3). The second pair of working springs record the moment of the force applied about the transverse axis — transverse d.c.g. (d.c.g. II, bridge II in Fig. 3). The tensosensitive bridges taking up the longitudinal and transverse moments of force are each composed of four resistance tensometers ($R_1$–$R_4$, d.c.g. I and $R_5$–$R_8$, d.c.g. II). All the arms of the bridge, connected as in a difference circuit, are working arms.

Standard resistance tensometers of 0·03 mm diameter constantan wire with a base of 45 mm and resistance of 250 Ω are applied, by means of BF-2 adhesive with subsequent polymerization, to the upper and lower planes of the working springs. The bridges are supplied from a generator with a voltage of 6 V with a frequency of 5 kc/s. The signal is taken from across the diagonal of the bridge, one end of which is earthed. The bridges are balanced by means of balancing devices incorporated into the

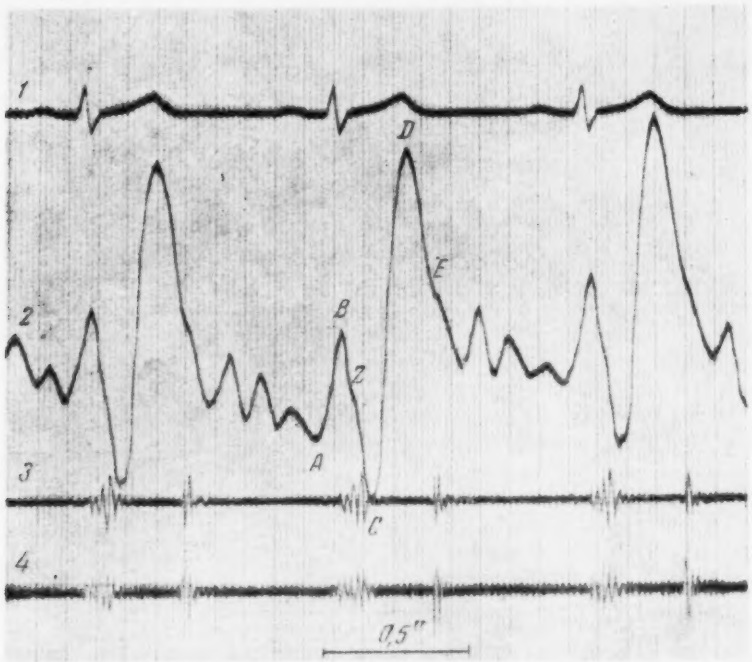


FIG. 4. Dynamocardiogram of normal subject recorded on loop oscillograph by a DKG-01 type (2) dynamocardiograph Letters indicate main spikes of dynamocardiogram. In upper part of figure electrocardiogram (1) in lower part, phonocardiogram (3 and 4).

amplifier unit (the active component, by the potentiometers $R_{12}$ and $R_{21}$ "roughly" and by potentiometers $R_{13}$ and $R_{22}$ "accurately"; the reactive component by the padders $C_1$ and $C_3$ "roughly" and by the potentiometers $R_{14}$ and $R_{23}$ "accurately"). Since recording of the longitudinal and transverse dynamocardiograms is carried out in turn the balancing devices are built in one constructional whole with gauged potentiometers.

As experience shows, segment CD of the longitudinal d.c.g. (Fig. 4) on average corresponds to a moment of about 2000 g.cm. A moment equal to 500 g.cm. is used for calibration. The calibration moment is produced artificially by displacing a spring weighing 50 g a distance 10 cm from the centre along the cover of the pick-up instrument. From this it is possible to estimate the relative elongation of the gauge-transducer λ and the relative change in the resistance of the arm of the bridge Er for the

calibration moment 500 g.cm. The relative elongation of the gauge-transducer applied to the upper plane of a spring fixed at one end is determined from the relation [13]:

$$\lambda = \frac{\Delta P}{EW}$$

where $W = bh^2/6$, the moment of resistance of the springs to bending. To determine the value of the change in the load $P$ the calibration moment is converted into the change of the reactions developing at the ends of the spring. The supporting reactions change; one of them diminishes and the other increases. The difference in the supporting reactions before and after moving the load 10 cm is about 6 g per spring, hence $\lambda = 0{\cdot}36 \times 10^{-6}$. For low values of $\lambda$ it may be considered that the relative compression of the transducer located in the lower plane of the working spring is equal to $\lambda$.

The relative change in the resistance is determined [14] from the equality:

$$E_r = \frac{\Delta R}{R} = S\lambda$$

where $S = 2$ the coefficient of tenso-sensitivity. Consequently, the change in resistance of one transducer is $\Delta R = 2 \times 250 \times 0{\cdot}36 \times 10^{-6}\ \Omega$ and the value of the calibration signal ($U$)

$$U_{sig} = \frac{U_{supply}\,\Delta R}{R_{arm}} = \frac{6 \times 180 \times 10^{-6}}{250} = 4{\cdot}5 \times 10^{-6}\ \text{V}.$$

Since each arm of the bridge is a working arm, the total signal in the diagonal is equal to $18 \times 10^{-6}$ V. This value of the signal determines the circuits of the electronic units of the instrument.

The signal, the value of which is determined by the "unbalance" of the tenso-sensitive bridge selected by means of switch $B_4$ (Fig. 3), is applied to the primary winding of the input transformer $Tr_1$. From the secondary step-up winding of this transformer the signal is supplied to a three-stage narrow band amplifier tuned to a frequency of 5 kc/s. The first stage is in the left half of the valve $L_1$ and is connected into a resistance-capacitance circuit. In the second stage assembled as in a transformer circuit ($Tr_2$ in Fig. 3) and tuned to a frequency of 5 kc/s the right half of valve $L_1$ is used. Between the second and third stages amplification regulators are included. The stepwise regulation of amplification is carried out by means of switch $B_3$ and smooth regulation by potentiometer $R_{43}$. The third stage is also arranged as a transformer circuit ($Tr_3$) and works into a phase-sensitive detector ring in which semiconductor diodes $D_1$–$D_4$ of the D7 Zh type and the smoothing resistances $R_{61}$–$R_{64}$ are used. The commutating voltage is supplied into the diagonal of the bridge of the ring detector from winding $Tr_4$. From the central points of the windings of the transformers $Tr_3$ and $Tr_4$ the demodulated signal is applied via filters $R_{70}$, $R_{71}$, $C_{25}$, $C_{26}$, $C_{28}$ and $C_{30}$ to the d.c. amplifier arranged in the valve $L_3$. From the d.c. amplifier the signal is fed to the control grids of the output stage ($L_4$ and $L_5$), connected as a symmetrical cathode-follower circuit, the load of which is the loop galvanometer of the recording instrument.

For precise balancing of the tenso-sensitive bridges the signal is additionally amplified by the right-hand triode of valve $L_2$, detected by the diode $D_5$ (type D7G) and supplied to the balance indicator, the milliammeter $IP_1$. The output indicator $IP_2$ together with potentiometer $R_{76}$ serves to adjust the loop galvanometer when recording.

Valves $L_7$, $L_8$, $L_9$ and $L_{10}$ form a voltage stabilizer which supplies the anode circuits of the amplifier and the generator (+200 V). The coefficient of stabilization is about 800.

The master oscillator, valve $L_6$ is transformer coupled and tuned to a frequency of 5 kc/s. The voltage supply to the tenso-sensitive bridges is fed from the secondary windings of transformer $Tr_4$. The commutating voltage at the ring detector is supplied from a special winding of the same transformer. All the units of the instrument are connected by detachable cables.

## TECHNICAL CHARACTERISTICS OF INSTRUMENTS

(1) The sensitivity of the instrument is such that with a change of the moment acting on the recording instrument of 500 g.cm. the output current on twofold amplification and shunted by a resistance

of 6 Ω is equal to 10 mA, which corresponds to a deviation of the light spot of the loop galvanometer of 35 mm when using a vibrator of the MOV-2 type No. 5.

(2) The frequency characteristic of the amplifier is linear over a frequency range of 0–100 c/s with a non-uniformity not exceeding 3 dB.

(3) The instrument is fed from the a.c. mains with a voltage of 127 or 220 V at a frequency of 50 c/s.

(4) With change of the supply voltage within limits of +5 to —15 per cent of the normal value the sensitivity of the instrument changes by less than 5 per cent.

(5) The power consumed by the instrument is about 80 W.

(6) The overall size of the amplifier unit is 360×320×230 mm.

(7) The overall size of the supply unit is 422×225×240 mm.

(8) The weight of the instrument (together with the carriage, recording instrument and the housing of the recording instrument) is about 35 kg.

Testing of the apparatus has shown that it ensures qualitative recording of dynamocardiograms making possible clear recording of all the spikes for temporal and amplitude analysis of the curves so obtained (Fig. 4). The instrument is simple and convenient to use and therefore can be used both in laboratories and under clinical conditions. The dynamocardiograph of this design can be used both independently and in combination with the electrocardiographs and other instruments for recording cardiac functions.

VOL.
5
1960

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# INVESTIGATION OF THE ACTIVITY OF BIPOLARS OF TWO TYPES BY THE METHOD OF ULTRA-VIOLET CYTOPHOTOMETRY *

I. A. UTINA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
5
196

ELECTROPHYSIOLOGICAL investigations carried out by means of the microelectrode technique have demonstrated the presence in the frog retina of bipolars of two types reacting differently to light stimulation [1]. The response of the bipolars of both types is shown in Fig. 1. The upper curves $b_1$ reflect the activity of the bipolars of the inner zone, the lower curves $b_2$ the activity chiefly of those of the outer zone. The former, both on switching on and switching off of light, give a slowly developing protracted oscillation; the second, a short oscillation on switching off of light and an oscillation of opposite direction on switching on. In accordance with these differences, a different reaction is also noted to flickering light. In the averaged curves it is clear that for frequent flickers (in this experiment 10 c/s) the rhythmical oscillations in potential are given only by bipolars of the outer zone.

Morphologically, all the bipolars in the frog retina are very similar. Only by certain methods of staining (Heedenhain's iron-haematoxylin method and silver) is it sometimes possible to observe two well-distinguishable layers of staining (Fig. 2). The ultra-violet method of cytophotometry makes it possible to verify experimentally the presence in the frog retina of bipolars of two types.

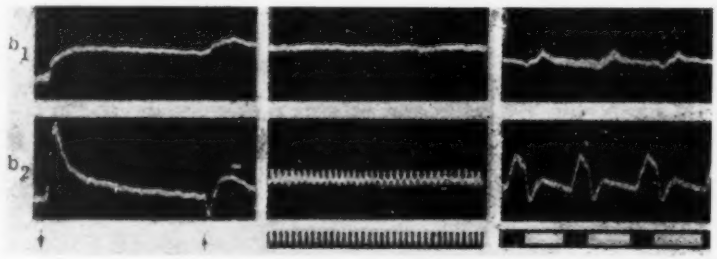


FIG. 1. Electrical response of different layers of the frog retina to flickering light. Recording by microelectrode located at a depth ~ 100 μ from the vitreous humour; $b_1$, recording between microelectrode and vitreous humour (bipolars of inner zone); $b_2$, recording between microelectrode and sclera (chiefly, bipolars of outer zone). Curves on left, "off" response (↓) and "on" response (↑) to light; centre, to frequent flickers (~ 10 c/s); on right, to infrequent flickers (~ 1 c/s). (Oscillograms obtained by A. L. Byzov).

The work of Brodskii *et al.* and other workers [2–7] has demonstrated direct correspondence between the activity of the nerve cells including the ganglion cells of the retina) and the RNA content of their cytoplasm. Thus, in the ganglion cells of the frog retina the RNA level is increased on stimulation with flickering light since only in these conditions do the cells give rhythmic discharges of pulses.

* Biofizika **5**: No. 5, 626–627, 1960.

The RNA in the cytoplasm of the ganglion cells on constant illumination remains at the same level as in the dark [8].

In view of these findings it is interesting to use RNA as an indicator of the activity of the bipolar cells of the frog retina in different conditions of light stimulation. It is obvious that in the case of the bipolars, by selecting a certain the light stimulation one can produce conditions in which only the bipolars of one type or simultaneously, both types, will be active or both types inactive.

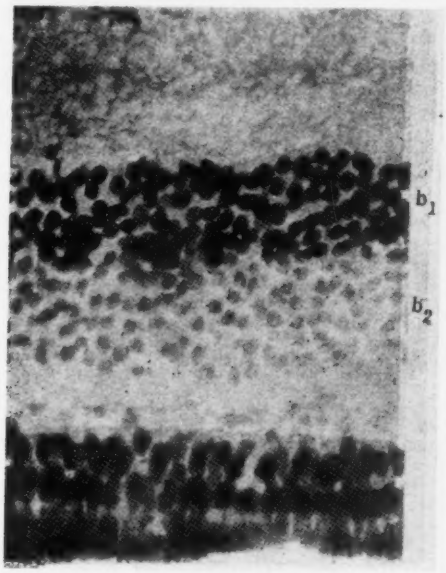


FIG. 2. Microphotographs of frog retina. Layer of bipolars: $b_1$, inner zone and $b_2$ outer zone. Staining by Bil'shovskii,s silver method. Objective ×40, ocular ×10.

In the dark the changes in the potential are absent in the bipolars of both zones and consequently a uniformly low RNA level can be expected in their cytoplasm. With flickering light of a frequency of about 4 c/s (such a frequency according to Byzov [1] is quite sufficient to make the cells of the inner zone of the bipolars stop functioning) only the bipolars of the outer zone function and the amount of RNA in their cytoplasm must obviously be considerably raised. Finally, with considerably less frequent flickering (1–0·5 c/s) rhythmical variations in potential are recorded in the bipolars of both zones (Fig. 1, right-hand curves) and an increase in RNA can be expected in all the bipolars.

On the basis of these ideas, experiments were carried out as follows.

Batches of the frogs *Rana ridibunda* of the same catch were placed in the dark for 24 hr. All the animals, in conditions of weak red illumination, were injected with a curare-like substance, diplacin, to render them motionless in order to eliminate any movements of the eye which could lead to changes in illumination of the retina. Some of the frogs were left in the dark, some placed for illumination with flickering light (~ 4 c/s or 0·5 c/s). Flickering light was obtained by means of a rotating disk interrupting a light beam from the illuminator (~ lx on the retina). The need to illuminate the animals as a whole (and not the isolated preparations of the eyes as is usually done in electrophysiological investigations) and the possibility of work with diplacin has been demonstrated by the work of Brodskii and Nechayeva [3]. The material was fixed 1 hr after the start of illumination.

For the quantitative estimation of RNA in the cytoplasm of the bipolars the ultra-violet method of photographic cytophotometry was used. The method of treatment and analysis have previously

been described in the papers of Brodskii and Nechayeva [2, 9, 10]. The RNA content in each variant of the experiment was determined as the average of measurements of 50–100 individual cells.

For measurement we chose bipolar cells sufficiently distant from the middle of the layer in order not to confuse the cells of different zones. The horizontal cells, the amacrine cells and any cells resembling them were not used for measurement.

The material obtained is presented in the form of a diagram in Fig. 3.

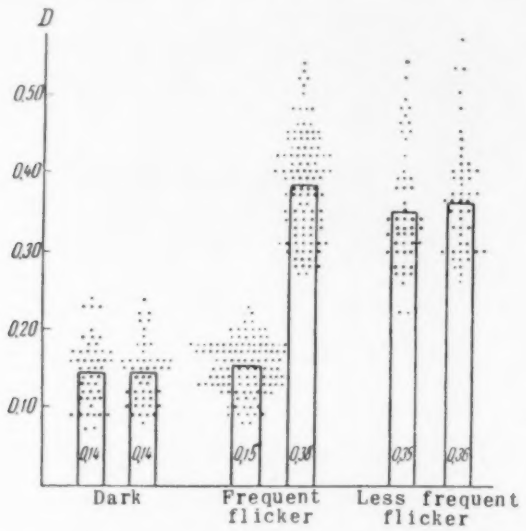


FIG. 3. Optical density of RNA (D) in the bipolars in various conditions of illumination. Time of action of light — 1 hr. (I) Dark; (II) Frequent flickering light; (III) Less frequent flickering light. Columns (and numerals) mean values; dots, density of RNA in individual cells (thickness of section 5 μ, λ = 265 mμ).

As Fig. 3 shows, the columns depict the mean values of the optical density of RNA from 50–100 cells of the bipolars of each type; in the dark the amount of RNA in the cytoplasm of both layers was practically identical. However, on illumination with frequent flickering light (~ 4 c/s) the amount of RNA considerably increased (more than double) only in the cytoplasm of the bipolars of outer zone. For less frequent flickering (1 c/s) the RNA content in the cytoplasm of the bipolars of both zones was increased (as compared with the content in the dark).

Hence, results of cytochemical investigations are in complete agreement with the predictions based on electrophysiological data, confirming the notion of the existence in the frog retina of bipolars of two types with different functional states.

The author wishes to thank A. L. Byzov, N. V. Nechayeva and V. Ia. Brodskii for their valuable advice and assistance in the work.

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* There is a translation into English of this work.

OL.
5
960

# γ-UNIT FOR CHRONIC IRRADIATIONS IN RADIOBIOLOGICAL EXPERIMENTS*

A. V. BIBERGAL'

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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DEVELOPMENT of radiobiological research requires elaboration of a specialized technique of irradiation of experimental objects.

One of the important trends is investigation of the sequelae of prolonged chronic irradiation at comparatively low dose rates. For this purpose in work with plants a special type of γ-unit is used in the form of a container-reservoir from which γ-rays (usually from $^{60}Co$) emerge as from a point source. Such an arrangement produces a dose field changing in the radial direction in inverse proportion to the square of the distance. The advantages of a γ-unit of this type is the possibility of easy selection of the desired dose rate by simple comparison of the distance and the value of activity of the source. For the irradiation of small objects or objects of small dimension in the radial direction of the dose field (single plants) the γ-unit with a point source is the best device for experimentation.

However, on irradiation of large living moving objects such a method cannot ensure strict experimental conditions since the object will be irradiated unequally.

To achieve uniformity of irradiation the object must be placed at a sufficiently great distance from the point source, this necessitates a very large area for experimentation. In a number of cases this is inconvenient, especially when the object has a considerable length in the radial direction of the apparatus.

The degree of inequality of the dose field between points $a$ and $b$ at distances $R_a$ and $R_b$ from the point source may be expressed by the relation:

$$\frac{P_a - P_b}{P_b} 100\% = \delta \qquad (1)$$

where $P_a$ and $P_b$, the dose rates at the corresponding points. Taking into account (1) and also the ratio

$$P = \frac{K_\gamma M}{R^2},$$

* Biofizika **5**: No. 5, 628–630, 1960.

where $K$ is the gamma constant and $M$, the activity of the source in mg. equiv. radium, we easily obtain:

$$R_b - R_a = \Delta_1 = [\sqrt{1 + 0{\cdot}01 \times \delta} - 1] \sqrt{\frac{K_\gamma M}{P_a}} \tag{2}$$

$$R_a = \frac{\Delta_1}{\sqrt{1 + 0{\cdot}01\,\delta} - 1} \tag{3}$$

From the given permissible degree of inequality $\delta$ the activity of the source M and the dose rate $P_a$ a permissible width of the object ($R_b$—$R_a$) is determined directly from (2) and the distance $R_a$ from expression (3) for the given values $\delta$ and $R_b$—$R_a$. In those cases in which it is necessary to submit large animals in cages or fish in aquaria to chronic irradiation, it is rational to use a γ-unit with a volume

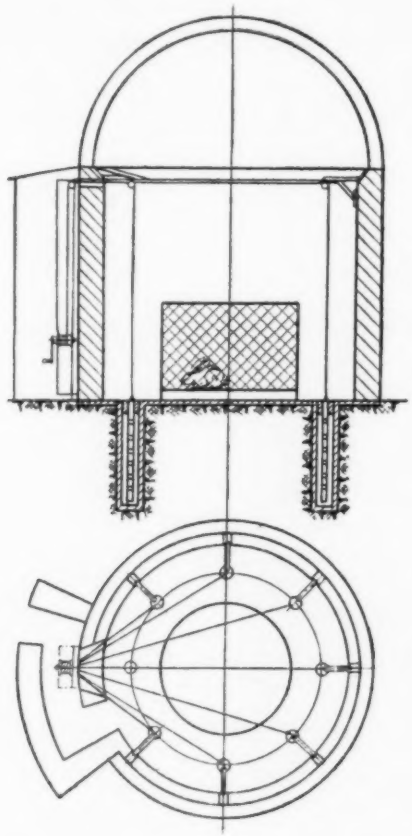

FIG. 1.

emitter, consisting of many point or linear sources arranged around the object of irradiation. Such an apparatus for the irradiation of animals was designed under the guidance of the Institute of Biophysics of the U.S.S.R. Academy of Sciences in the Institute of Pathology and Therapy of the U.S.S.R. Academy of Medical Sciences of the town of Sukhumi in 1958.

A similar device for irradiation of marine organisms is planned for the Biological Station of the U.S.S.R. Academy of Sciences in the town of Sevastopol.

The apparatus for chronic irradiation of animals (monkeys, rabbits, etc.) is in the form of a cage with a diameter of about 1·5 m, around which are located eight sets of point sources. Each set of sources is enclosed within a common vertical tube and is arranged in such a way that the activity of the individual sources increases from the upper end of the tube downwards. All eight tubes with the sources can be simultaneously raised and lowered, by means of a system of rods, in such a way that for irradiation one, two or several sources with increasing activity can be used as desired. This makes it possible to regulate the dose rate in several steps. Selection of the distances to the sources and the value of the activity of the latter makes it possible to set any limits on the change in dose rate. In particular, in starting up the apparatus each of its rods is charged with four sources and the dose rate in the cage can be changed from 0·01 r/hr to 1·2 r/hr in four steps.

OL.
5
960

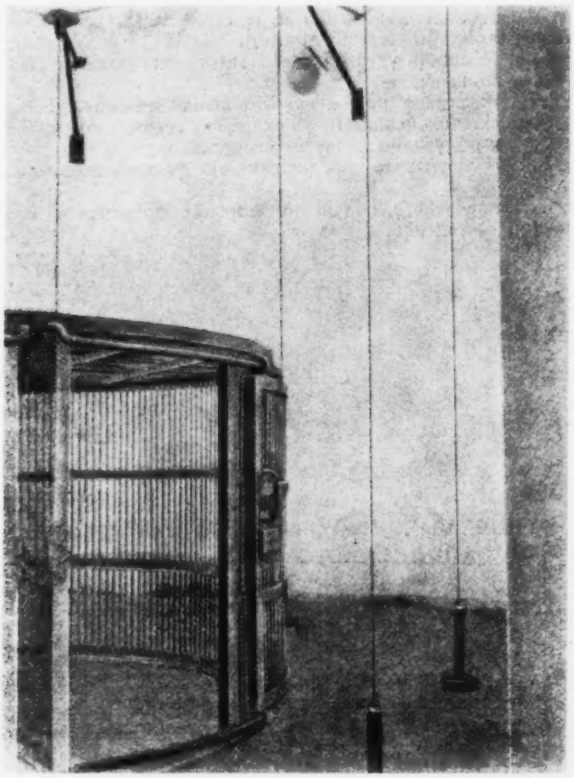

FIG. 2.

The dose field of the apparatus was calculated in such a way that the dose rates in the horizontal plane, halfway up the cage would vary by not more than 20 per cent. The dosimetric check (made by K. S. Kalugin, U.S.S.R. Academy of Medical Sciences) confirmed this calculation.

At individual sites in the cage (in the corners at top and below) the dose rate deviates from the mean value outside the limits indicated above but the regions of the maximal and minimal values of the dose rate are a small part of the volume and in the greater part of the volume of the cage changes in dose rate are within the limits of the calculated values.

Figure 1 shows a schematic plan of the apparatus and Fig. 2 a photograph of the inner part. The total area occupied by the apparatus is 12·6 m², diameter about 4 m, the overall activity of the sources in the device is less than 4 g equiv. radium.

An apparatus with a single point source and a dose field varying in the radial direction would, in order to achieve the same degree of uniformity of irradiation of animals in cages with a total width of 0·5 m and for the same maximal limit of the dose rate, have to have a diameter of about 13 m and an activity of about 50 g equiv. radium. The area of the apparatus correspondingly ought to be about 130 m², i.e. 10 times greater.

A similar problem arises in the design of γ-units for experimental chronic irradiation of marine organisms in an aquarium. In this case the object of irradiation is the entire aquarium and in calculating the dose field of irradiation account must be taken of absorption and scatter of radiation in the total mass of water.

The apparatus for chronic irradiation of marine organisms in the aquarium will be described in a subsequent communication.

VOL
5
196

# FRACTION OF RADIATION EMERGING FROM A HOLLOW CYLINDRICAL SOURCE FILLED WITH ABSORBING SUBSTANCE*

YE. YE. KOVALEV and D. P. OSANOV

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IN a number of cases it is necessary to define the fraction of radiation emerging from a hollow cylindrical filled with absorbing substance. For example, in determining the yield of γ-radiation from cylindrical sources in many cases it is necessary to compute the dose, from the radio-active material, adsorbed by the walls of the source. The radiation is to a certain extent adsorbed by the substance of the source. The fraction of γ-radiation emerging from the hollow cylindrical source depends on the height and radius of the source, the distance to the source, the energy of the adsorbed γ-rays and the atomic number of the absorbing substance.

Another possible example is a hollow cylindrical emitter intended for sterilization of various objects by continuous radiation [1]. In this case, the γ-radiation emerging from the hollow cylindrical emitter is partially absorbed in the object being irradiated and the fraction of emerging radiation is a measure of the effective use of the radiation of the source.

Calculations are made below of the fraction of radiation emerging from a hollow cylindrical source filled with absorbing substance in relation to the relative dimensions of the source $k = h/R$, the distance to the given point $p = b/R$ and the parameter $\mu R$. Here $\mu$ is the coefficient of absorption of γ-rays in the substance filling the hollow cylindrical source. The following assumptions are made; (a) each element of the cylindrical surface $dS$ is an isotropic monoenergetic emitter; (b) the absorption of γ-rays in the substance filling the hollow cylindrical source is of exponential form; and (c) there is no absorption of radiation in the cylindrical wall containing the source.

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Using the cylindrical co-ordinates with a centre at point $O$ (Fig. 1), we can write an expression for the fraction of radiation emerging from the hollow cylindrical source filled with absorbing substance in the form:

$$f = \frac{\int_0^H dz \int_0^{\arccos \frac{R}{b}} \frac{d\varphi}{R^2+b^2-2Rb\cos\varphi+z^2} + \int_0^H dz \int_{\arccos \frac{R}{b}}^{\pi} \frac{e^{-2\mu R \frac{(R-b\cos\varphi)\sqrt{R^2+b^2-2Rb\cos\varphi+z^2}}{R^2+b^2-2Rb\cos\varphi}}}{R^2+b^2-2Rb\cos\varphi+z^2} d\varphi}{\int_0^H dz \int_0^{\pi} \frac{d\varphi}{R^2+b^2-2Rb\cos\varphi+z^2}}$$

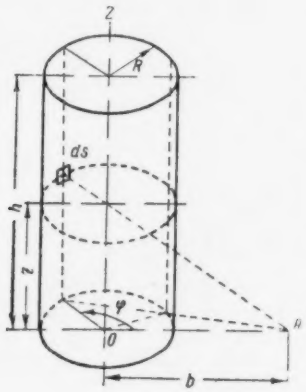


FIG. 1. Calculation of fraction of radiation emerging from hollow cylinder.

Here, the fraction of radiation $f$ is determined for point $A$ located in the plane of the lower base of the cylinder. The numerator in expression (1) corresponds to the yield of radiation from the hollow cylindrical source filled with absorbing substance and the denominator to the yield of γ-radiation from the hollow cylindrical source. The first integral in the numerator corresponds to the yield of γ-radiation

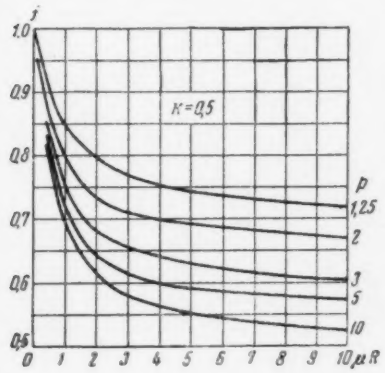


FIG. 2. Fraction $f$ as a function of $\mu R$ for $k = 0{\cdot}5$.

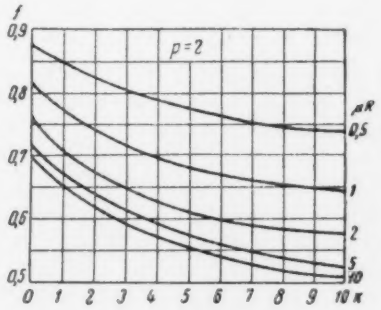


FIG. 3. Fraction $f$ as a function of $k$ for $p = 2$.

of the "visible" part of the surface of the hollow cylinder not shielded by absorbent, the second to the yield of γ-radiation from the remaining "invisible" part of the surface of the hollow cylinder shielded by absorbent. The double integral in the denominator may be reduced [2] to the tabulated class one elliptical integrals $F(\varphi, \alpha)$. In our case

$$\varphi = \text{arct an } h/R \text{ and } \alpha = \text{arc sin } \sqrt{\frac{2Rb}{R+b}}$$

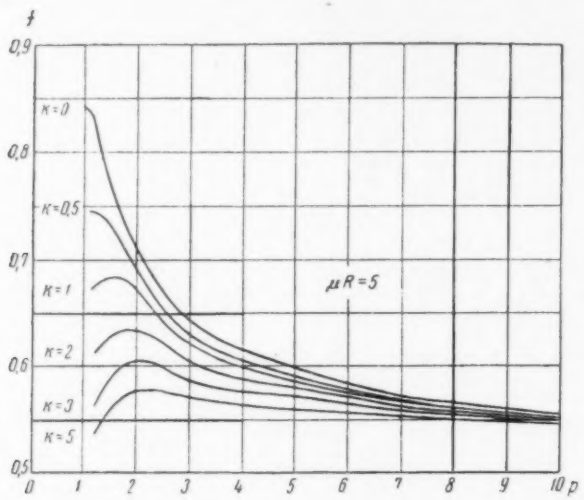


FIG. 4. Fraction $f$ as function of $p$ for $\mu R = 5$.

Allowing for this possibility and using the relative values $p$ and $k$, we can rewrite expression (1) in the following form:

$$f = \frac{1+p}{\pi \cdot F(\varphi, \alpha)} \left\{ \int_0^k dn \int_0^{\arccos 1/p} \frac{d\varphi}{1 + n^2 + p^2 - 2p\cos\varphi} + \right.$$

$$\left. + \int_0^k dn \int_{\arccos 1/p}^{\pi} \frac{e^{-2\mu R \frac{1-p\cos\varphi}{1+p^2-2p\cos\varphi}\sqrt{1+n^2+p^2-2p\cos\varphi}}}{1 + n^2 + p^2 - 2p\cos\varphi} \right\} \qquad (2)$$

The double integrals in (2) are indefinite. Calculation of this integral for various values of $p$, $k$ and $\mu R$ was made on the "Strela" electronic computer. The results of the calculations of the fraction of radiation for a series of values of $p$, $k$ and $\mu R$ are given in Table 1.

Figure 2 shows the relation between the fraction $f$ and the parameter $\mu R$ for $k = 0{\cdot}5$ and various values of $p$. It is clear that with increase in $\mu R$, the fraction $f$ diminished and diminished more rapidly with greater values of $p$. For a sufficiently high value of $\mu R$, as the calculations show, the fraction $f$ equals the ratio of the dose contributions from the "visible" and the entire hollow cylindical surfaces. It is obvious that when $\mu R \to 0$ for any values of $p$ and $k$, then $f \to 1$. The relation between $f$ and the parameter $k$ for $p = 2$ and various values of $\mu R$ is shown in Fig. 3.

It is clear that for increase in $k$, the fraction $f$ decreases and reaches values close to 0·5 when $\mu R$ is sufficiently great. Reduction in $f$ with increase in $k$ for constant values of $p$ and $\mu R$ may be explained by the increased rôle of the so-termed oblique rays, their influence being all the greater the larger $\mu R$.

TABLE 1. FRACTION OF RADIATION $f$ EMERGING FROM HOLLOW CYLINDRICAL SOURCE FILLED WITH ABSORBING SUBSTANCE

| $p$ | $\mu R$ for $k = 0{\cdot}05$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 0·5 | 1·0 | 2·0 | 3·0 | 5·0 | 7·0 | 10·0 |
| 1·25 | 1·000 | 0·901 | 0·847 | 0·794 | 0·768 | 0·743 | 0·730 | 0·720 |
| 1·5 | 1·000 | 0·887 | 0·828 | 0·776 | 0·753 | 0·732 | 0·720 | 0·709 |
| 2·0 | 1·000 | 0·860 | 0·790 | 0·730 | 0·707 | 0·692 | 0·681 | 0·670 |
| 3·0 | 1·000 | 0·832 | 0·751 | 0·679 | 0·652 | 0·627 | 0·615 | 0·603 |
| 5·0 | 1·000 | 0·811 | 0·718 | 0·641 | 0·612 | 0·590 | 0·582 | 0·572 |
| 7·0 | 1·000 | 0·801 | 0·704 | 0·622 | 0·592 | 0·565 | 0·553 | 0·545 |
| 10·0 | 1·000 | 0·794 | 0·695 | 0·612 | 0·579 | 0·551 | 0·538 | 0·525 |
| $p$ | $\mu R$ for $k = 1{\cdot}0$ | | | | | | | |
| | 0 | 0·5 | 1·0 | 2·0 | 3·0 | 5·0 | 7·0 | 10·0 |
| 1·25 | 1·000 | 0·870 | 0·801 | 0·735 | 0·705 | 0·676 | 0·662 | 0·652 |
| 1·5 | 1·000 | 0·866 | 0·797 | 0·738 | 0·712 | 0·684 | 0·676 | 0·664 |
| 2·0 | 1·000 | 0·849 | 0·775 | 0·712 | 0·689 | 0·672 | 0·662 | 0·652 |
| 3·0 | 1·000 | 0·829 | 0·745 | 0·683 | 0·646 | 0·621 | 0·608 | 0·596 |
| 5·0 | 1·000 | 0·809 | 0·717 | 0·639 | 0·611 | 0·588 | 0·580 | 0·570 |
| 7·0 | 1·000 | 0·800 | 0·703 | 0·622 | 0·591 | 0·564 | 0·553 | 0·545 |
| 10·0 | 1·000 | 0·794 | 0·694 | 0·612 | 0·579 | 0·551 | 0·537 | 0·525 |
| $p$ | $\mu R$ for $k = 2{\cdot}0$ | | | | | | | |
| | 0 | 0·5 | 1·0 | 2·0 | 3·0 | 5·0 | 7·0 | 10·0 |
| 1·25 | 1·000 | 0·826 | 0·740 | 0·664 | 0·633 | 0·616 | 0·606 | 0·595 |
| 1·5 | 1·000 | 0·828 | 0·747 | 0·680 | 0·652 | 0·628 | 0·614 | 0·604 |
| 2·0 | 1·000 | 0·823 | 0·740 | 0·672 | 0·648 | 0·632 | 0·623 | 0·615 |
| 3·0 | 1·000 | 0·816 | 0·728 | 0·656 | 0·627 | 0·603 | 0·592 | 0·578 |
| 5·0 | 1·000 | 0·805 | 0·711 | 0·635 | 0·605 | 0·579 | 0·571 | 0·561 |
| 7·0 | 1·000 | 0·798 | 0·701 | 0·620 | 0·588 | 0·563 | 0·551 | 0·543 |
| 10·0 | 1·000 | 0·793 | 0·693 | 0·610 | 0·578 | 0·550 | 0·536 | 0·525 |

Figure 4 shows the relation between $f$ and $p$ for $\mu R = 5$ and various values of $k$. For low values of $p$, the fraction $f$ passes through a maximum which is particularly well in evidence for $k = 1$–5. With further increase in $p$, the fraction $f$ falls off reaching a value close to 0·5. This character of the dependence of $f$ on distance may be explained as follows. The rise in $f$ with increase in $p$ is related to the lessening of the rôle of the oblique rays which is particularly marked at high values of $k$ (relative height of cylinder). On passing to high values of $p$, the fraction $f$ changes as the ratio of the dose contributions from the "visible" and entire cylindrical surfaces and this ratio falls off with increase in $p$.

In the absence of repeated scatter in the absorbing substance the results given above are correct. Approximate allowance can be made for this by means of analytical representation of the accumulation factors [3]. It is obvious that repeated scatter of γ-radiation in the absorbing substance leads to decrease in the fraction of γ-radiation emerging from the hollow cylindrical source.

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# LETTERS TO THE EDITOR

## DOSIMETRY OF RADIATION OF PULSED X-RAY TUBES WITH AUTOELECTRONIC EMISSION AND THE POSSIBILITY OF THEIR USE IN RADIOBIOLOGY*

A. A. ZOTIKOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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VOL
5
196

IT has recently been demonstrated [1, 2] that ionizing radiations at a very high dose rate (over $10^7$ rad/sec) and of extremely short duration (less than 2 μsec) give unusual radiobiological and radiochemical effects. Suitable apparatus needs to be designed for investigations in this field [3, 4].

It was thus of interest to explore the possibilities of using pulsed X-ray tubes with autoelectronic emission from the cold cathode in radiobiology. We tested IA-320 tubes, similar in design to those devized by Slack [5] and established the following dosimetric characteristics; dose per pulse, dose rate per pulse, duration of pulse and quality of radiation.

The tube was connected into the Arkad'ev—Marks circuit depicted in Fig. 1. The principle of action of this circuit is described in many text-books on high-voltage techniques [6]. To discharge the circuit, a trigger device, specially prepared by us, giving pulses with a voltage of 10 kV was used. The voltage supply to the tube is $U_0 = 180$ $kV_{max}$ and the discharge capacity $C = 0{\cdot}007$ μF. Special attention was paid to reducing the external resistance, especially the inductive one, which affects the ouput of X-radiation. The tube works as an aperiodic system.

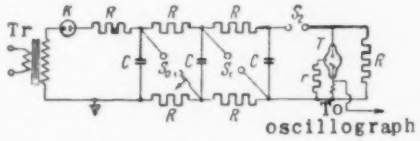


FIG. 1. Basic circuit diagram of pulsed tube (Arkad'ev—Marks circuit). (Tr) High voltage transformer; (K) Kenotron; (R, r) Aqueous resistances; (C) 0·022 μF condensors; ($S_0$, $S_1$, $S_2$) Spark gaps; (T) Pulsed tube.

The radiation dose was measured by an ionization method and the pulse duration by the oscillograph. For the purpose of measuring the latter, a special high-voltage pulse oscillograph was assembled in the Laboratory of Radiobiology (Prof. Shekhtman's team) according to the scheme devized in the high-voltage laboratory of the E.N.I.A.N. The block diagram of this oscillograph is given in Fig. 2. A 26I square-pulse generator was used to check on the absence of distortion in the shape of the pulses. The LO-249 cathode-ray tube was supplied with a voltage of up to 6 kV. The pulses of current were photgraphed on fluororapid film. The mean duration of the radiation pulse estimated from the oscillograms was $\tau = 1$ μsec.

* Biofizika **5**: No. 5, 634–636, 1960.

Measurement of the dose rate per pulse from a pulsed X-ray tube with autoelectronic emission offers particular difficulties since we have to deal with very high radiation intensities (over $10^6$ r/sec) with a relatively low value of dose per pulse (of the order of a few roentgens) and a low frequency of pulse sequence (a few pulses per min). We measured the dose rate at a distance of 14 cm from the anode of the tube with a specially designed dosimeter. Since for dose measurements at very high radiation intensities not only is the high voltage of the field in the chamber of importance, but also small inter-electrode distances [7]. We used a special ionization chamber with small clearance ($d = 2$ mm). The voltage supply to the chamber was V = 1000 V which ensured a field voltage of 5000 V/cm. Higher values of field voltage in the chamber are dangerous owing to the appearance of "shock ionization". Because of the relatively low value of the dose per pulse, to increase the accuracy we measured the dose of several consecutive pulses. Repeated measurements gave an average dose per pulse of the order of 5 r. The degree of saturation in the ionization chamber was evaluated from the Langeven—Boag formula [8]:

$$f = \frac{1}{u} \ln (1+u)$$

where $u = 1000 \dfrac{rd^2}{V}$. Here, r is the dose per pulse in roentgens, $d$, the distance between electrodes in cm and $V$, the voltage in the chamber in volts.

Substituting the numerical values for $r$, $d$ and $V$ in this formula we get:

$$u = 1000 \frac{5 \times 0{\cdot}04}{1000} = 0{\cdot}2 .$$

From the graph given in reference [8] for $u = 0{\cdot}2$, the efficacy of collection of ions $f = 91$ per cent. Hence, the mean dose per pulse at a focal distance of 14 cm is 5·5 r.

With a pulse duration of $\tau = 10^{-6}$ sec, we obtained a mean dose rate per pulse $P = 5{\cdot}5 \times 10^6$ r/sec. Hence, an extremely high dose rate is given by the IA-320 type pulsed X-ray tube with autoelectronic emission.

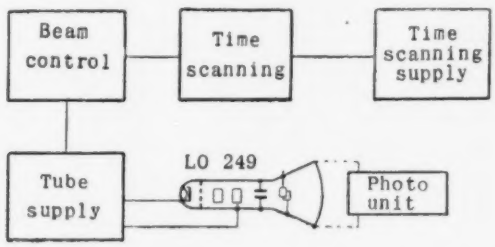


FIG. 2. Block circuit diagram of high voltage pulse oscillograph.

Next we determined the quality of the radiation by the method of absorption in aluminium filters. To exclude the small scatter in the values of the dose per pulse, we measured simultaneously the dose with two ionization chambers (one of which measured the unfiltered radiation) and then took the ratio $I_1/I_2$ of the readings for each chamber. The results of the measurements are given in Fig. 3. The linear coefficient of absorption determined from these findings is $\mu = 8{\cdot}6$ cm$^{-1}$. From the Table (see [9]) we find the effective wavelength $\lambda_{eff} = 0{\cdot}585$ Å, which corresponds to $U_0/3$.

Thus, the radiation from the pulsed X-ray tube with autoelectronic emission is softer than that of the ordinary X-ray tube, a fact to bear in mind in radiobiological experiments. This is in agreement with the findings of Funfer [10] who, however, worked with a lower voltage ($U_0 = 40$ kV) and confirms the assumption of Popov [11] on the reduced hardness of radiation of pulsed tubes with autoelectronic emission.

A defect of these tubes is their short service life. After passing several hundred pulses the inner side of the glass envelope of the tube becomes coated with a metallic deposit, a result of vaporization of the material of the anode. The mirror formed leads to defocusing of the electronic beam and a sharp drop in the dose rate of the X-radiation.

The investigations show that the pulsed X-ray tubes with auto-electronic emission from the cold cathode but designed for higher voltages (1–1·5 × $10^6$ V), as exhibited at the All-Union Industrial Exhibition of 1958, will probably give a dose per pulse of the order of ~ 100 r at a dose rate of $10^8$–$10^9$ r/sec and may be successfully applied in radiobiology.

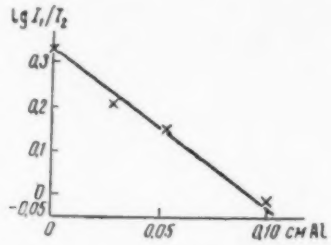


FIG. 3. Graph of radiation absorption in aluminium. Ordinates, intensity of radiation in relative units; abscissa, thickness of aluminium filters, cm.

In connexion with the use in biology of the new and very sensitive method of research — paramagnetic resonance [12–14] — pulsed tubes with autoelectronic emission in conjunction with electron accelerators [4] may be a convenient source of radiation in studies on rapidly-developing processes and, in particular, the kinetics of free radicals. The production of such complex devices, built for the first time in the world by the Institute of Chemical Physics of the U.S.S.R. Academy of Sciences [15], opens up a wide field of activity in investigations of primary radiobiological processes.

In conclusion, the author wishes to express his gratitude to Prof. Ia. L. Shekhtman for guidance and assistance in the work.

## SUMMARY

(1) A determination has been made of the dosimetric characteristics of IA-320 type pulsed X-ray tubes with autoelectronic emission from the cold cathode; dose per pulse, dose rate, duration of pulse and quality of radiation were studied.

(2) It has been shown that high-vaccum tubes with autoelectronic emission give an extremely high dose rate (~ $10^7$ r/sec) with a pulse duration of the order of 1 μsec.

(3) It has been established that on determining the effective wavelength of radiation one should start from a value one-third of the maximum voltage applied to the tube, i.e. radiation of tubes with autoelectronic emission is softer than for ordinary tubes.

(4) It is assumed that tubes of this type, but designed for higher voltages, may be a convenient source of radiation of a microsecond duration in investigations of rapidly-developing primary radiochemical processes by means of the highly-sensitive paramagnetic resonance spectrometer.

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VOL.
5
1960

# DETERMINATION OF FREE RADICALS IN TISSUES OF RATS INOCULATED WITH SARCOMA 45*

I. K. KOLOMIITSEVA, K. M. L'VOV and L. P. KAIUSHIN

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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It has previously been shown that stable free radicals can be detected in the surviving tissues of healthy animals by the method of electron paramagnetic resonance [1]. It was of interest to see how a pathological state of the body affects the concentration of stable radicals in various metabolically-active tissues. In the view of some workers, an increase in the concentration of free radicals in the tissue may be a cause of tumorigenesis [2, 3].

## EXPERIMENTAL

The experiments were performed on male white rats weighing 200 g 1 month after inoculation with sarcoma 45. Both the control and tumour bearing rats were presented to us by Member of the Institute of Nutrition of the U.S.S.R. Academy of Medical Sciences, N. M. Fil'chagin to whom the authors would like to express their gratitude. At the time of the experiments the tumours measured $40 \times 30 \times 15$ mm.

The animals were killed by decapitation, the organs removed and 70 mg fresh homogenized tissue used for investigating the e.p.r. spectra. The Table gives the results of measurements of the amplitude of the first derivative of the signal (with uniform signal width, the amplitude depends on the concentration of unpaired electrons).

It follows from the results that free radicals are detected in the tumour tissue although in minimal concentrations as compared with all the other organs investigated of the tumorous rats. The number of free radicals in the liver and brain of the tumorous and healthy rats was uniform. In the spleen of the tumour-bearing rats, the content of free radicals was slightly increased. This increase is of interest in connexion with the known anti-carcinogenic properties of the spleen [4–6] and points to special changes in the functions of the spleen in animals with a tumour.

* Biofizika **5:** No. 5, 636–637, 1960.

TABLE 1. AMPLITUDE OF FIRST DERIVATIVE OF E.P.R. SIGNAL FROM THE ORGANS OF TUMOUR BEARING AND HEALTHY RATS

| Organ | Tumour-bearing rats | | Healthy rats | |
|---|---|---|---|---|
| | No. of tests | Amplitude | No. of tests | Amplitude |
| Tumour | 9 | 14±4 | — | — |
| Brain | 7 | 18±3 | 7 | 19±6 |
| Liver | 8 | 27±6 | 7 | 28±8 |
| Spleen | 7 | 21±4 | 7 | 13±5 |


## SUMMARY

(1) The e.p.r. method has been applied to investigate the number of free radicals in the surviving tissues of rats: in inoculated sarcoma 45, brain, liver and spleen.

(2) It has been shown that the number of free radicals detected in the tumour itself is less than for the other organs of tumorous rats.

(3) The number of free radicals in the brain and liver of the rats with tumour did not differ from that in the brain and liver of healthy rats.

(4) In the spleen of the tumorous rats, the content of free radicals was increased, this is possibly related to the anti-carcinogenic functions of this organ.


We express our deep gratitude to A. M. Kuzin and G. M. Frank for their interest in the work.

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* There is a translation into English of this work.

# PROBLEM OF THE USE OF VITAMIN C IN RADIATION SICKNESS*

A. V. S. IUSIPOV

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CONFLICTING findings appear in the literature on the effect of vitamin C in radiation sickness. We decided to study the effect of ascorbic acid in relation to its time of administration.

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In the experiments on 88 rabbits with whole-body irradiation with a dose of 500 r, a study was made of the effect of ascorbic acid on the course of the acute radiation sickness, the blood sugar and lactic acid contents and the sugar curves in the galactose test.

In the 32 control animals, irradiated but not receiving vitamin C, it was established that the changes in the indices of carbohydrate metabolism in acute radiation sickness have a phase character; the most marked shifts are observed in the first hours after irradiation, changes are almost completely absent in the "latent" period and appear again in the peak period.

Administration of ascorbic acid before irradiation (25 rabbits) had an unfavourable influence. There was a greater increase than in the controls, in the lactic acid content of the blood and distortion of the form of the sugar curves. In the experimental group 7 of 25 animals died in the first 2 weeks after irradiation as against 3 of 32 in the control group. The same negative result was shown by administration of vitamin C immediately after irradiation (experiments on 12 rabbits).

In the next experimental series (19 animals) the ascorbic acid was administered 20 hr and later after irradiation. It was found to have a distinctly favourable effect; the lactic acid content of the blood (on testing at the peak of the disease) was normal, the sugar curves were as at the start of the experiment and no deaths were noted in the first 2 weeks after irradiation.

In experiments on 70 white rats we studied the effects of vitamin C on the liver glycogen content. The glycogen was determined by the Feulgen-Shabadash and Pfluger methods.

Two hours after whole-body irradiation with a dose of 600 r, the liver glycogen of the irradiated rats was higher (3489 mg %) than for the unirradiated rats (1691 mg %). We considered this increase in liver glycogen in the first hours after irradiation was one of the manifestations of an early protective reaction (glycogenesis due to the products of disturbed metabolism).

Administration of ascorbic acid before irradiation inhibited this defence reaction as indicated by the less marked accumulation of liver glycogen (only up to 2144 mg %). Administration of vitamin C for 5 days before irradiation, as for a single dose before irradiation, suppressed the defence reaction of the liver to irradiation. On examination 3 days after irradiation the content of liver glycogen was down to 746 mg %. Still less glycogen (616 mg %) was contained in the liver of the animals receiving vitamin C just before irradiation. As a result of administration of ascorbic acid 1, 2 and 3 days after irradiation there was a considerable (3·5 times) increase in the content of liver glycogen (up to 2594 mg %).

This is evidence of a distinctly marked favourable effect of administration of vitamin C after irradiation.

The results reported here may be used in studies of the primary mechanism of the action of radiation on the body.

A practical conclusion to be drawn is the need to prohibit use of ascorbic acid for prophylaxis and emergency therapy in acute radiation sickness. Vitamin C may be recommended in the therapy of acute radiation sickness only after disappearance of the symptoms of the primary reaction, i.e. in the "latent" period and at the peak. Normalization of the metabolic processes may be promoted by making good the deficit in vitamin C (within definite limits of radiation doses).

## DISCUSSION

# STEADY STATES AND THERMODYNAMIC THEORY OF EXCITATION OF LIVING TISSUES*

A. K. PRITS

Leningrad Herzen State Teaching Institute

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VOL
5
196

THE present paper is an attempt to apply the thermodynamics of open systems to one of the most important problems of physiology — that of excitation of living tissues and, in particular, to the problem of muscular contraction. It is not the intention of the author to consider the molecular mechanism of this contraction. The article is simply a phenomenonological description of the activity of muscle tissue as a mechano-chemical machine controlled by the central nervous system.

We shall consider the elementary nerve-muscle unit since the contraction of the muscle is a complex total effect of the contraction of its component muscle fibres working incoherently. This ensures the reliablity of the work of the entire muscle as a whole [1].

It has long been known that the magnitude of the contraction of the muscle fibre is determined by the frequency of the nerve impulses entering it. But the activity of the muscle fibre also comes about as a result of the energy of the substances entering it from without. These substances as a result of a whole cycle of coupled chemical reactions within the fibre are decomposed with liberation of energy. It is most important to establish the links between the expenditure of energy in the muscle tissue per unit time, the rate of the chemical reactions within the fibre and the frequency of the nerve impulses entering this fibre.

Since the rate of the chemical reactions is dependent on the rate of entry of substances into the nerve tissue, this also signifies the establishment of the link between the frequency of the nerve impulses and the metabolic rate.

## STEADY STATE OF LIVING ORGANISMS

The investigations of Prigogine and Wiame [2] showed that the living organism is in the steady state. The equilibrum is written in the form of constancy of entropy of the living system:

$$\frac{dS}{dt} = \frac{d_i S}{dt} + \frac{d_e S}{dt} = 0_i . \qquad (1)$$

The living system constitutes an open system exchanging matter and energy with the surrounding space. $d_iS/dt$ is the increase in entropy due to chemical reactions proceeding irreversibly within the open system and $d_eS/dt$ is the inflow of negative entropy from without due to metabolism. We shall consider the muscle fibre as a separate open system in a state of metabolism. As with any living system it takes up more highly organized substances and discards less organized ones. This degradation of the substances received from without sustains the invariability of the structure of living organisms.

We shall consider that the equality (1) applies to muscle tissue and we shall write it in the form of a variation of the theorem of Prigogine for the existence of steady states [3].

* Biofizika **5**: No. 5, 639–643, 1960.

Under the action of external forces tending to take the living system out of the steady state, processes occur in it to compensate the changes produced by these forces.

This principle is known in biology as homeostasis and is a modification of the principle of Le Chatelier. This means that the living system is able to regulate the rate of exchange with the surrounding medium changing it in conformity with change in the surrounding environment. This suggests that the living system is able to receive information on these changes and order the work of its parts accordingly.

It is the responsibility of the central nervous system to ensure that equality (1) is fulfilled for the muscle tissue.

The quantity of energy given off by the muscle tissue per unit time as a result of the whole cycle of coupled chemical reactions which take place irreversibly within the tissue (quantity of energy received from without as a result of metabolism) is defined by the equality [4]:

$$T \frac{d_i S}{dt} = Al, \tag{2}$$

where $A = \sum_i \mu_i \nu_i$ is the chemical affinity representing the sum of the products of the chemical potentials $\mu_i$ and the corresponding stoichiometric coefficients $\nu_i$ and $I$ is the rate of these reactions and $T$, the absolute temperature.

The law of conservation of energy for open systems may be written in the form [4]:

$$T \frac{d_i S}{dt} = \frac{dE}{dt} + p \frac{dv}{dt} - h \frac{dM}{dt}, \tag{3}$$

where $dE/dt$ is the rate of change in the internal energy in the muscle fibre, $p$, the pressure within the fibre, $dv/dt$, the rate of change in the volume of the tissue, $h$, the enthalpy and $dM/dt$, the rate of change in the mass of the system.

We shall consider that the mass and volume of the muscle tissue are not changed during contraction and the last two terms can then be discarded.

The law of conservation of energy for open systems is then written in the form:

$$T \frac{d_i S}{dt} = \frac{dE}{dt}. \tag{4}$$

## MECHANISM OF MUSCULAR CONTRACTION AND HILL'S EQUATION

The muscle tissue structure is of such a form that it ensures direct conversion of chemical energy into mechanical work.

In the muscle fibre a distinction is made between the ordinary nucleated protoplasm known as sarcoplasm and the differentiated protoplasm or "inoplasm". In the skeletal muscles of vertebrates the sarcoplasm is concentrated at the periphery of the muscle fibre and does not take part directly in contraction of the fibre. Contraction of the muscle fibre is determined by change in the inoplasm. We shall proceed from a model of the structure of inoplasm proposed by Huxley [5] and based on X-ray electronomicroscopy data. According to this model, the inoplasm is represented in the form of threads of actin and myosin, entering the one into the other and joined together by transverse bonds. The change in the elastic energy of the muscle fibre and hence the performance of mechanical work is determined by the change in number of the transverse bonds joining the threads of actin and myosin.

This idea was advanced much earlier by Frenkel' [6].

The formation of transverse bonds between actin and myosin occurs spontaneously since it is linked with reduction in chemical energy and leads to liberation of heat. Rupture of the transverse bonds, and consequently, recovery of the original structure, is dependent on the disintegration of adenosine triphosphate (ATP). Breakdown of ATP as shown by a number of findings [7] is ensured by the enzymatic properties of the actinomyosin complex formed at the junctions between actin and

myosin. Therefore, it is natural to assume that the rate of disintegration of ATP is proportional to the rate of formation of the transverse bonds between actin and myosin.

The rate of change in the internal energy of unit volume of a muscle fibre of length $l_0+\Delta l$, where $l_0$ is the normal length and $\Delta l$, the magnitude of stretching of the tissue (in the natural state, muscle tissue is always stretched), is determined by the expression [6]:

$$-\frac{\Delta E}{\Delta t}=\left[\frac{1}{2}E_0\left(\frac{\Delta l}{l_0}\right)^2-\varepsilon N\right]\frac{\Delta n}{\Delta t}, \tag{5}$$

where $E_0$ is the modulus of elasticity, $\varepsilon$, the reduction in chemical energy as the result of formation of one bond, N, the number of threads of actin and myosin bound by transverse bonds and $\Delta n/\Delta t$, the rate of change in the number of bonds. Since in the state of rest $\Delta l$ is constant, the elastic energy per bond $\frac{1}{2}E_0\left(\frac{\Delta l}{l_0}\right)^2$ is a constant. This means that the mechanical work $U_0$ performed by the muscle fibre in the formation of one bond is a constant value

$$U_0=-\frac{1}{2}E_0\left(\frac{\Delta l}{l_0}\right)^2=\text{const.}, \tag{6}$$

and $B=\varepsilon N/U$ is greater than unity since the muscle constantly gives off heat. Therefore, expression [5] may be rewritten as:

$$\frac{\Delta E}{\Delta t}=(U_0+BU_0)\frac{\Delta n}{\Delta t}=D\frac{\Delta n}{\Delta t}U_0=D\frac{\Delta U}{\Delta t} \tag{7}$$

where $D=B+1$ and $\Delta U/\Delta t=U_0\cdot\Delta n/\Delta t$ is the rate of change of the complete elastic energy. Since in the state of rest the amount of heat given off per unit time is a constant value, it follows from (7) that $D\left(\frac{\Delta U}{\Delta t}\right)_0=\text{const.}$, i.e. $\left(\frac{\Delta n}{\Delta t}\right)_0=\text{const.}$, or that the rate of formation (and consequently the rate of rupture) of the transverse bonds between the threads of actin and myosin is a constant value in the state of rest.

The constancy of the rate of change in the elastic energy of the muscle tissue in the state of rest may be equated with the presence in the resting muscle of a constant force $a$ performing the same amount of work in unit time.

$$\left(\frac{\Delta U}{\Delta t}\right)_0=U_0\left(\frac{\Delta n}{\Delta t}\right)_0=ab, \tag{8}$$

where $a=\frac{1}{2}E_0\left(\frac{\Delta l}{l_0}\right)\Delta n$ and $b=\frac{1}{l_0}\frac{\Delta l}{\Delta t}$, the relative rate of contraction of the fibre.

If muscle tissue passing into the excited state develops the mechanical power necessary to raise the load $p$ with a relative rate of contraction $v$, then:

$$\frac{\Delta U}{\Delta t}=(p+a)(v+b), \tag{9}$$

$$\frac{\Delta E}{\Delta t}=D(p+a)(v+b). \tag{10}$$

The expressions (9) and (10) were obtained experimentally by Hill [8]. It is clear from (4) and (10) that with constant temperature the equality

$$Al=D(p+a)(v+b). \tag{11}$$

applies.

## MECHANO-CHEMICAL CHARACTERISTICS OF THE PROCESS OF EXCITATION OF MUSCLE TISSUE

In the state of rest of the muscle fibre at constant temperature the product $AI$ is a constant value. And since the chemical affinity is always constant, this means that the rate of the coupled chemical reactions $I$ within the tissue in a state of rest is a constant value.

The rate of any chemical process may be expressed by the formula [9]:

$$I = ce^{-\frac{\Delta F}{kT}}, \tag{12}$$

where $c$ is the constant of the rate of the reaction, $k$ the Boltzmann constant, $T$ the absolute temperature and the equality

$$\Delta F = F_{ak} - F_0 \tag{13}$$

is the free energy of the activiation being the difference between the free energy of the activated state $F_{ak}$ and of the ground state $F_0$ (see Fig. 1).

It is clear from (12) that the requirement of constancy of the rate of reaction $I$ in the state of rest at constant temperature signifies constancy of the free energy of activation $\Delta F$. Therefore, we shall express the rate of the chemical processes within the tissue in a state of rest by the formula:

$$I_0 = ce^{-\frac{\Delta F_0}{kT}}. \tag{14}$$

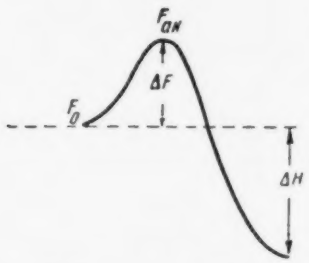


FIG. 1. Activation barrier. ($F_0$) Free energy of ground state; ($F_{ak}$) Free energy of activated state; ($\Delta F$) Energy of activation; ($\Delta H$) Thermal effect of reaction.

From the mechanical point of view, the state of rest of the muscle fibre may be characterized by absence of the load $p$ and rate of contraction $v$. Therefore, the state of rest of the muscle fibre will be determined by the following mechano-chemical equation:

$$AI_0 = Dab. \tag{15}$$

The mechanical expression of the excited state is the presence of load $p$ or a rate of contraction $v$ or both together. This according to (12) and (11) requires an increase in the rate of the chemical processes $I$. Therefore, the value of excitation $\alpha$ of the muscle fibre may be defined by the difference between the rate of the chemical processes $I$ in the excited state and in the resting state:

$$\alpha = A(I - I_0) \tag{16}$$

or according to (11) and (15),

$$\alpha = D[(p+a)(v+b) - ab]. \tag{17}$$

Expression (16) gives the chemical characterization of the process of excitation and expression (17) the mechanical.

## FUNDAMENTAL EQUATION FOR THE ACTIVITY OF MUSCLE FIBRE

Passage into the excited state, as already indicated, is characterized by an increase in the rate of the chemical processes occuring within the tissue. It is plain from (12) that at constant temperature this may be achieved only by change in the free energy of activation. On the other hand, it is well known that the passage into the excited state of the muscle fibre in natural conditions occurs only under the influence of nerve impulses.

We shall start from the commonly-accepted interpretation of the nerve impulse as carrier of information [10]. It may be assumed that as a result of receiving the nerve impulse a definite mechanism is established with the result that the system increases its negative entropy. This leads to decrease in the free energy of activation and thereby to increase in the rate of the chemical reactions. If as a result of receiving nerve impulses the system acquires $\Delta S$ negative entropy then the free energy of activation is expressed by the equality:

$$\Delta F = F_{ak} - F_0 - T\Delta S = \Delta F_0 - T\Delta S, \tag{18}$$

and the rate of the chemical processes according to (12) and (14) is written:

$$I = ce^{-\frac{\Delta F_0 - T\Delta S}{kT}} = I_0 e^{\frac{\Delta S}{k}}. \tag{19}$$

It is well known that the process of transmission of nerve impulses is discontinuous and rhythmical. The value of the nerve impulse, if we are to judge by the amplitude of the action currents recorded from the nerve fibre, is constant. It may thus be assumed that the amount of negative entropy received by the system from without as the result of receiving one nerve impulse is constant. We shall designate it as $S_0$. Then the amount of negative entropy received in 1 sec is equal to

$$\Delta S = \omega S_0, \tag{20}$$

where $\omega$ is the frequency of nerve impulses and $S_0$ has the dimensions cal. sec/degree. Consequently, expression (19) with respect to (20) may be rewritten:

$$I = I_0 e^{\frac{\omega S_0}{k}}. \tag{21}$$

Equation (21) shows that the rate of the chemical processes within the fibre is determined by the frequency of the incoming nerve impulses. Therefore, the value of the excitation may also be characterized by the frequency of nerve impulses — which incidentally is what is happens in physiology.

With reference to (21) equation (11) may be rewritten in the form:

$$AI_0 e^{\frac{\omega S_0}{k}} = D(p+a)(v+b). \tag{22}$$

But $AI_0 = Dab$ (see (15)). Therefore, (22) may be rewritten as:

$$e^{\frac{\omega S_0}{k}} = \left(\frac{p}{a}+1\right)\left(\frac{v}{b}+1\right). \tag{23}$$

Equation (23) defines the law governing the work of the muscle fibre as a mechano-chemical machine. It links the frequency of the nerve impulses entering the fibre across the synapse (we can disregard transformation due to the work of the synapse) with the mechanical power developed by this muscle fibre on contracting.

It is clear from (8) and (9) that $ab = U_0\left(\frac{\Delta n}{\Delta t}\right)_0$ and $(p+a)(v+b) = U_0\frac{\Delta n}{\Delta t}$.

Therefore expression (23) may be rewritten in the form:

$$\frac{\Delta n}{\Delta t} = \left(\frac{\Delta n}{\Delta t}\right)_0 e^{\frac{\omega S_0}{k}}. \tag{24}$$

Equation (24) shows that the rate of formation (and hence of destruction) of the transverse bonds between actin and myosin is defined by the frequency of the incoming nerve impulses. Since the process of excitation is of a discontinuous character and the period of excitation is replaced by a period of complete inexcitability, it may be assumed that the processes of formation and destruction of the transverse bonds between the threads of actin and myosin alternate with each other.

Expression (23) reflecting the logarithmic dependence of the frequency of nerve impulses on the mechanical power developed by the muscle tissue is undoubtedly related to the approximately logarithmic nature of the Weber-Fechner law and awaits interpretation on the basis of physico-chemical investigations of the activity of the central nervous system.

Expression (23) has been confirmed experimentally. For $v = 0$ and $p \gg a$ direct proportionality is obtained between the frequency of the nerve impulses and the value of the load $p$. For values of load comparable with $a$, deviation from this proportionality is, in fact, observed.

VOL.
5
1960

## SUMMARY

The present work gives a thermodynamic theory for the excitation of living tissues. The law for the work of the muscle fibre as a mechano-chemical machine controlled by the nervous system is derived from the steady-state principle of living systems formulated by Prigogine and Wiame. The paper discussed the mechanism of muscular contraction based on the latest experimental findings.

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VOL
5
196

# EFFECT OF DENATURATION AND COMPLEXING WITH PROTEINS ON THE MAGNETIC PROPERTIES OF NUCLEIC ACIDS*

SHENG PEI-KEN, L. A. BLYUMENFEL'D and A. E. KALMANSON

Institute of Chemical Physics, U.S.S.R. Academy of Sciences, Moscow
Bakh Institute of Biochemistry, U.S.S.R. Academy of Sciences, Moscow

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IN previous studies [1–3] new anomalous magnetic properties of nucleic acids and certain artificially prepared complexes of nucleic acids with proteins were discovered and investigated. These magnetic properties consist in the appearance of wide (600–1200) oersted asymmetrical magnetic resonance signals of very high integral intensity and also the appearance of positive static magnetic susceptibility displaying distinct saturation with a rise in the voltage of the magnetic field. The previous investigations have clearly shown that these effects fundamentally depend on the structure of the nucleic acids and are not related to paramagnetic or ferromagnetic contamination.

In the present work we have studied in detail the change in the magnetic properties of nucleic acids during denaturation and also, on complexing in various conditions with proteins. Because the structure and properties of DNA are now much better known than the corresponding characteristics of RNA, most of our work was done with DNA. Recently, papers [4–6] have appeared showing that native nucleic acids and ribonucleoprotein contain firmly bound metallic ions—both paramagnetic (Cr, Mn, Fe, Ni, Cu) and nonparamagnetic (Mg, Al, Ca, Zn, Sr, Cd, Ba) (*sic*). RNA from various sources contains one metallic atom per 50 nucleotides and DNA one per 150. These metals are not impurities and form part of the organic structure of the nucleic acids, apparently in the form of chelates. As such, these metals cannot account for the observed effects. However, serious attention must be paid to their presence in considering the nature of the effect. In the present article we shall not deal with the physical nature of the effect observed. We would only note that from the point of view of magnetic properties the compounds tested cannot be considered as paramagnetic. This is indicated by the anomalous temperature dependence of the resonance signals and the static susceptibility and also the effects of saturation. In the strict sense of the word they cannot be considered ferromagnetic, since despite the presence of saturation the residual magnetic moment of the preparations is equal to zero. Likewise, they cannot be assigned to the anti-ferromagnetics. Hence, the compounds tested and also a number of synthetic polymers of definite structure [7, 8] constitute a new class of active magnetic substances the properties of which do not fit into the existing classification.

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## MATERIAL AND METHODS

1. *Measurement of e.p.r. spectra*

The measurements were made on a EPR-2 IKhF radiospectrometer with high frequency modulation of the magnetic field [9]. A specially designed device made it possible to record the spectra in a range of mangetic fields from 0–6000 oersted. The measurements were carried out at a frequency of 9300 Mc/s. To evaluate the intensity of the signal it was compared with the signal of an iron-containing polymer, kindly made available to us be V. P. Parin, recorded in the same conditions. The intensity of the signal of this polymer was cerfully calibrated by us against a standard sample of the stable free radical of diphenyl picryl-hydrazyl. Therefore, we shall arbitrarily evaluate the intensity of the signal by the number of unpaired electrons per gramme preparation. This evaluation is arbitrary because, as the experimental data as a whole show, in our case it was a matter of comparing collective spin magnetism with the intensity of the e.p.r. signal of a normally paramagnetic substance, which strictly speaking, is not legitimate. The figures we give have to be interpreted as follows; the number of unpaired electrons of the normally paramagnetic substance which would give an e.p.r. line of the same integral intensity.

The accuracy of determination of the relative intensity of the signals was not less than 5 per cent. Since it was noted that the intensity of the signals of the complexes fell off with time after their preparation, all the measurements were carried out not later than six hours after obtaining the preparation. The e.p.r. spectra were always recorded at room temperature and all are given in the form of the first derivative of the absorption line.

2. *Starting materials*

We had at our disposal two nucleic acids, RNA and DNA, and also two proteins, ovalbumin (OA) and tropomyosin (TM). The RNA (from yeasts), a Burroughs Wellcome preparation, was kindly placed at our disposal by Professor Sayo Tian-tsin of the Institute of Biochemistry of the Academy of Sciences of the Chinese People's Republic, to whom the authors wish to express their sincere gratitude. The DNA (L. Light & Co.) did not completely dissolve in water. It is possible that this was due to the presence of small amounts of nucleoproteins. When investigating denaturation we used a preparation not treated in any way. For complexing with proteins the two nucleic acids were suspended in a large excess of distilled water and filtered. After lyophilization the nucleic acids, now easily soluble in water, were used for complexing. The intensity of the e.p.r. signal of DNA thereupon diminished. Ovalbumin was isolated by L.V. Vozvyshaeva from hen eggs. Tropomyosin was extracted from the rabbit muscle by the method of Bailey [10]. All these starting materials were preserved in the form of lyophilized preparations.

3. *Heating DNA in the dried from*

The samples were heated on a water or sand bath. The same sample was heated at a definite temperature and its e.p.r. spectra recorded every 30 min.

4. *Heat denaturation of DNA in solution*

A 0·4% solution of DNA was heated on a water bath at a definite temperature for a definite time, after which it was dried by lyophilization. To exclude the effect of dissolving and lyophilization, the intensity of the signals was compared with the intensity of a dissolved, lyophilized but unheated preparation.

In order to observe the effect of NaCl on heat denaturation of DNA in solution, 0·4 per cent DNA solutions containing a variable amount of NaCl were heated at 100° for 7 min. The solutions were dialysed in the cold against water until the salt was completely removed, with subsequent lyophilization.

5. *Acid denaturation of* DNA

A 0·4% DNA solution was incubated at 37° for 3 hr with various amounts of hydrochloric acid then dialysed in the cold against water to remove the hydrochloric acid and then lyophilized.

6. *Preparation of complexes of nucleic acids and proteins*

General conditions; solutions of nucleic acid and protein were added to buffer solution of known pH. After shaking, the mixture was left in a cold room (0–2°C) and at the end of 20 hr centrifuged. The sediment was washed three time with a fixed volume of the corresponding dilute buffer solution, then dialysed in the cold against water until the inorganic salts were completely removed followed by lyophilization. The conditions for the individual complexes are given below.

1. RNA + OA complexes; at various pH values from 3·2–4·4, 8 ml N acetate buffer containing 5·6 per cent NaCl was added 21 ml of a 0·4 % solution of RNA and 21 ml of a 0·4% solution of OA.

At various weight ratios of RNA/OA a 0·4% solution of RNA and 0·4% solution of OA were added to 8 ml of the above-mentioned buffer (pH 4·0). The total volume of the solutions (apart from the buffer) was 42 ml.

The effect of the concentration of NaCl on complexing; to 8 ml N acetate buffer (pH 4·2) containing NaCl in various amounts was added 21 ml of a 0·4% solution of RNA and 21 ml of a 0·4% solution of OA.

2. DNA + TM complexes; at various pH values from 2·4–4·6, 10 ml phosphate-citric acid buffer (prepared from 0·2 M $Na_2HPO_4$. $2H_2O$ and 0·1 M citric acid) was added to 10 ml of a 0·2% solution of DNA and 20 ml of 0·2% solution of TM.

At various weight ratios of DNA/DM, 10 ml of the above-mentioned buffer (pH 2·8) was added to a 0·2% solution of DNA and 0·2% solution of TM. The total volume (apart from buffer) was 30 ml. The effect of the NaCl concentration on complexing; to 10 ml of the above-mentioned buffer (pH 2·8) containing NaCl in various amounts was added 22·5 ml of 0·2% solution of DNA and 7·5 ml of 0·2% solution of TM.

3. DNA + OA complex; at various pH values complexes were prepared in the same way as in (2).

At various weight ratios of DNA/OA complexes were prepared as in (2) but with the pH of the buffer 2·4 instead of 2·8.

4. RNA + TM complexes; at various pH values (pH ~3·8–5·4) 7·5 ml of phosphate-citric acid buffer (see 2) was added to 11·3 ml of 0·4% solution of RNA and 11·3 ml 0·4% solution of TM.

At various weight ratios of RNA/TM 7·5 ml of the above-mentioned buffer (pH 4·6) was added to 0·4% solution of RNA and 0·4% solution of TM. Total volume (apart from buffer) was 22·5 ml.

In the course of the present investigation a large part of the material was dried by lyophilization on our behalf by L. V. Nikiforova and D. M. Dmitrieva in the Bakh Institute of Biochemistry of the U.S.S.R. Academy of Sciences. The authors wish to thank them for their contribution to the work.

7. *Determination of the content of nucleic acid in the complexes*

For all the original material and the complexes derived from them an analysis was made for phosphorus by the colorimetric method [11]. The phosphorus content was 8·60 per cent in RNA, 9·08 per cent in DNA and 0·11 per cent in OA; TM did not contain phosphorus. An idea of the content of nucleic acid in the given complex may be gained from the content of phosphorus as compared with the starting materials.

## RESULTS

1. *Experiments with denaturation of DNA*

Fig. 1 shows the e.p.r. spectrum of the original DNA preparation. Intensity of the signal corresponds to $1{\cdot}8 \times 10^{21}$ unpaired electrons per gramme of preparation.

Fig. 2 represents the relation between the relative intensity of the e.p.r. signal and heating of the DNA in the dried form in air. As Fig. 2 shows, the intensity of the signal fell appreciably on heating both at 100° and at 150°. In the latter case the fall occurred

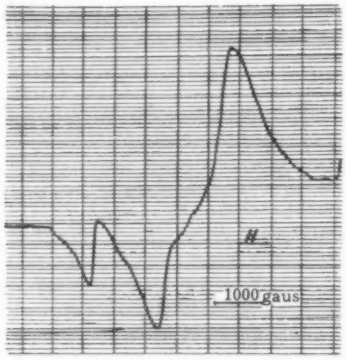


FIG. 1. E.p.r. spectrum of original DNA preparation.

more rapidly. It is quite clear that in the initial period after the start of heating the fall in intensity occurred extremely rapidly, the subsequent changes an hour after the start of the heating proceeded much more slowly. The impression is formed that in the DNA preparation there are at least two fractions of highly different stability.

Apparently, this is reflected in the incomplete insolubility of the preparation (see above). There are references to the inhomogeneity of DNA in the literature [12–15].

Fig. 3 depicts the results of investigation of the effect of 10-min heating of DNA solution at various temperatures on the intensity of the e.p.r. signals. There is information in the literature on the effect of heating at various temperatures on molecular weight (according to viscosity and the sedimentation coefficient) [16–22] and on the degree degree of structural stability (according to the hypochromicity) [15, 23–30] of DNA in solution. Both these parameters decrease with rise in temperature. As Fig. 3 shows, with a rise in temperature, the intensity of the signal rapidly fell, apparently in parallel with the degree of denaturation.

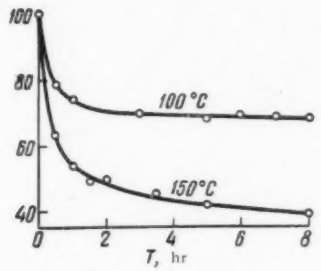


FIG. 2. Reduction in relative intensity of e.p.r. signal of DNA on heating in dried form. Ordinates, relative intensity.

Fig. 4 shows the relation between the intensity of the e.p.r. spectra of DNA and the time of heating of the solution at 80 and 100°C. As Fig. 4 indicates, during, the first 10 min of heating there was a sharp fall in intensity, then the intensity of the signals again rose, reaching 46·7 per cent after 20 min at 80°C and 37·3 per cent at 100°C. Further heating again led to a reduction in the signals. Such a complex kinetic relationship may be interpreted as the result of the partial transition of the nucleic acid during denaturation from one orderly form to another before final denaturation. As is known, some workers point to the presence of several forms of DNA and to the existence of transition between them under the influence of various conditions [31–33]. Other workers have also shown [19, 22, 34] that DNA preparations are not homogenous with respect to heat denaturation.

The protective action of weak concentrations of salt on denaturation of aqueous solutions of DNA has been described by a number of workers [25, 27, 28, 35–38]. Therefore, it was of interest to investigate the effect of NaCl on the stability of the e.p.r. signals on heating DNA in aqueous solution. The results of these experiments are given in Fig. 5. In this figure the relative intensities are expressed as percentage of the intensity of the signal of the original DNA. It is clear that a rise in concentration of NaCl from zero to 0·42 M led to a decreased effect of heating on the intensity of the signals. This, qualitatively, coincides with the findings in the literature on the stabilizing effect of NaCl. According to our findings (see Fig. 5) the stabilizing effect is maximal at a concentration of 0·42 M NaCl. Unfortunately, the effect of high concentrations

of NaCl (greater than 1 M) on the temperature behaviour of DNA solutions has not been investigated for the other methods in the literature [25–28].

In a special series of experiments we investigated acid denaturation of DNA. The results of these investigations are given in Fig. 6. The relative intensity is expressed as percentage of the intensity of the signal of the original DNA. It should be noted

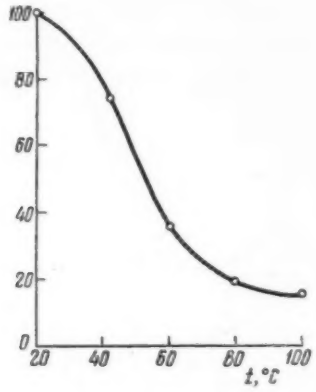


FIG. 3. Relative intensity of e.p.r. signals of DNA as a function of temperature of heating in solution for 10 min. Ordinates, relative intensity.

that at 37° the intensity of the e.p.r. signals considerably fell even for pure aqueous solution of DNA (without acid). As Fig. 6 shows, acid denaturation led to a sharp drop in the signals. As is known, acid denaturation is accompanied by changes in the DNA structure [39–43].

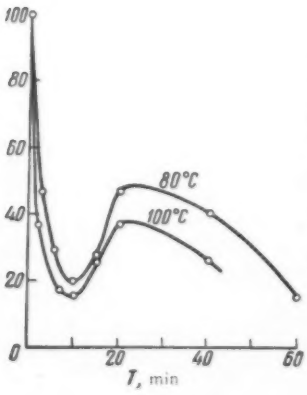


FIG. 4. Reduction in intensity of e.p.r. signals of DNA on heating of its solutions. Ordinates, relative intensity.

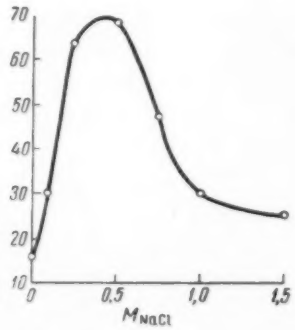


FIG. 5. Effect of NaCl on reduction of relative intensity of e.p.r. signals of DNA with 7-min heating at 100°. Ordinates, relative intensity.

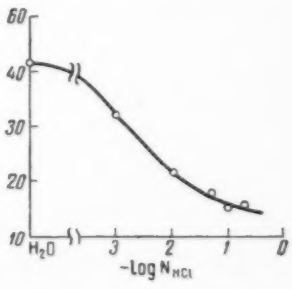


FIG. 6. Reduction in intensity of e.p.r. signals of DNA treated with HCl in solution at 37° for 3 hr. Ordinates, relative intensity.

2. *Experiments with complexes of nucleic acids and proteins*

Before formation of the protein complex, the OA and TM did not give e.p.r. signals. The RNA and DNA preparations used to prepare the complexes gave weak wide e.p.r. lines, the intensities of which corresponded to $1{\cdot}4 \times 10^{20}$ (RNA) and $1{\cdot}9 \times 10^{20}$ (DNA) unpaired electrons per gram. As a result of complexing, the intensity of the signals rose to $2{\cdot}6 \times 10^{21}$ unpaired electrons per gram of complex and on conversion to 1 gramme of nucleic acid to $5 \times 10^{22}$. Fig. 7 shows a typical e.p.r. spectrum for the RNA + OA complex containing 14·4 per cent RNA, the intensity of which corresponded to $2{\cdot}6 \times 10^{21}$ unpaired electrons per gramme.

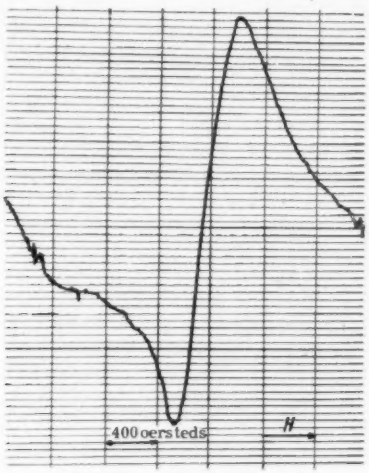


FIG. 7. E.p.r. spectrum of RNA + OA complex containing 14·4 per cent RNA intensity of which corresponds to $2{\cdot}6 \times 10^{21}$ unpaired electrons per gramme.

The width of the e.p.r. lines between the points of maximum slope was from 400 to 1000 oersteds for the complexes. The g factors of the point of maximum absorption were shifted from the g factor of the free electron to a value of 2·2–2·4. The maximum intensity was observed for the RNA + OA complexes. The intensity of the signals of the complexes diminished in the order: RNA + OA > DNA + TM > DNA + + OA > RNA + TM.

Unlike the original nucleic acids and proteins all the complexes obtained by us were insoluble in water. In all cases, apart from the RNA + OA complex after mixing the corresponding solutions the precipitate appeared at once. After centrifugation, insoluble complexes no longer precipitated from the supernatant fluid and the nucleic acids and proteins left behind in the solution after lyophilization did not give a wide e.p.r. line of higher intensity than the original nucleic acids. Thus, for example, on mixing 225 ml 0·2% solution of DNA and 75 ml 0·2% solution TM with 100 ml phosphate-citric acid buffer (pH 2·8), 172 mg DNA + TM complex was precipitated, containing 43·8 per cent DNA. The intensity corresponded to $1{\cdot}14 \times 10^{21}$ unpaired

electrons per gramme. After lyophilization of the supernatant solution a DNA and tropomyosin mixture was obtained containing 87·3 per cent DNA. The intensity corresponded only to $1{\cdot}11 \times 10^{20}$ unpaired electrons per gram.

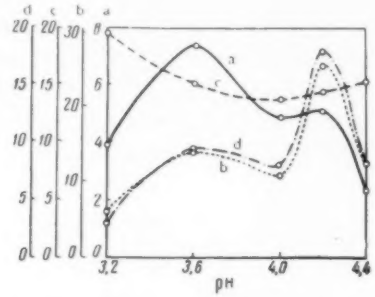


FIG. 8. RNA + OA complex obtained for various pH values. Ordinates, (a) yield of complex in mg, (b) intensity of signal in concentration of unpaired electrons per g $\times 10^{-20}$, (c) % nucleic acid content in complex, (d) intensity of signal on conversion to 1 g nucleic acid $\times 10^{-21}$.

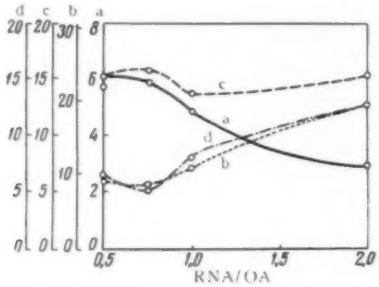


FIG. 9. RNA + complexes obtained at pH 4 and for various initial weight ratios of RNA and OA. Symbols as in Fig. 8.

The RNA complex with ovalbumin was obtained by mixing 315 ml 0·4% solution of RNA and 315 ml 0·4% solution of OA in 120 ml N acetate buffer (pH 4·2) containing 5·6% NaCl. After standing for 20 hr at 0–2°C, 85 mg of the RNA + OA complex precipitated containing 14·4 per cent RNA, the intensity of the signals of which corresponded to $2{\cdot}6 \times 10^{21}$ unpaired electrons per gram. After a further 147 hr there was an additional formation of 55 mg precipitate containing 5·2 per cent RNA with a signal intensity of $1–3 \times 10^{21}$ unpaired electrons per gram. After a further 190 hr 23 mg of a complex containing 3·2 per cent RNA with a signal intensity of $2{\cdot}7 \times 10^{20}$ unpaired electrons per gramme was precipitated. After lyophilization of the supernant, a mixture was obtained containing 52·8 per cent RNA not giving an e.p.r. signal.

It was noted that the composition of the complexes, their yield and the intensity of the signals observed depended essentially on the pH at which the complexes were obtained and on the ratios of concentration of nucleic acid and protein used for com-

plexing. The results of the relevant expertiments are shown in Figs. 8–15. As the figures indicate, it was not possible to find any general relationship for the yield and composition of complex and intensity of e.p.r. signal for all the cases considered. At the same time, for individual pairs a distinct parallelism (RNA + OA, DNA + TM) or lack of parallelism (DNA + OA) in the yield of the complex and the intensity

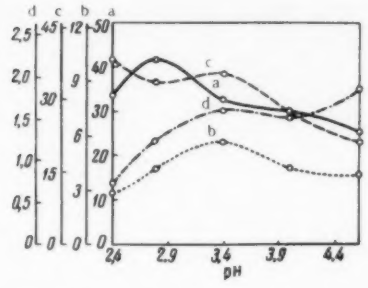


FIG. 10. DNA + TM complexes obtained for various pH values. Symbols as in Fig. 8.

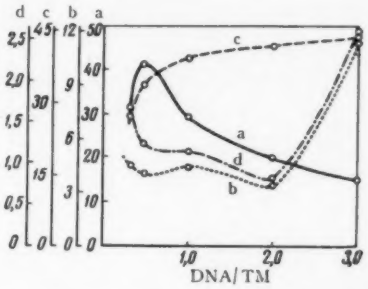


FIG. 11. DNA + TM complexes obtained for pH 2·8 and for various initial weight ration of DNA and TM. Symbols as in Fig. 8.

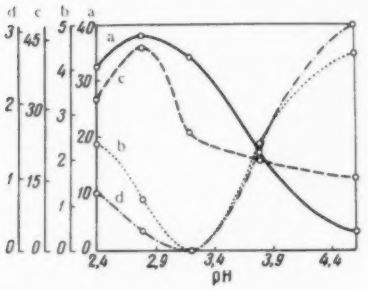


FIG. 12. DNA + OA complexes obtained for different pH values. Symbols as in Fig. 8.

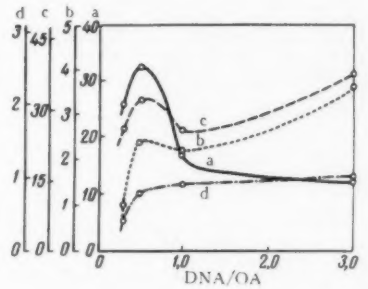


FIG. 13. DNA + OA complexes obtained at pH 2·4 and for various initial weight ratios of DNA and OA. Symbols as in Fig. 8.

of the e.p.r. signals could be observed. It is clear from the results that the intensity of e.p.r. signal does not bear a well-defined relation to the content of nucleic acid in the complex. This indicates that on complexing in various conditions the structure of the nucleic acid itself changes fundamentally. If the effect was merely due to the metals contained in the nucleic acid then between the intensity of the signals of the complex and the content of nucleic acid complete parallelism should exist.

In the literature the effect of electrolytes, in particular, NaCl, on the formation of complexes (their yield and composition) of nucleic acids with proteins has been reported [44]. We ascertained whether change in the NaCl concentration affects the magnetic properties and the results of these experiments are shown in Figs. 16 and 17.

It is clear from the findings that the concentration of NaCl exerts an appreciable effect on the magnetic properties of the complexes.

We noticed that the intensity of the e.p.r. signal of the dried complexes falls with time of storage of the dried preparations in air and *in vacuo*. On storage in air in certain cases, the intensity of the signal fell virtually to zero after a few weeks. There was

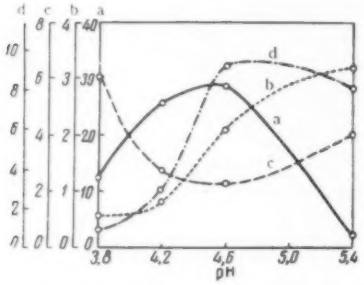


FIG. 14. RNA + TM complexes obtained for different pH values. Symbols as in Fig. 8.

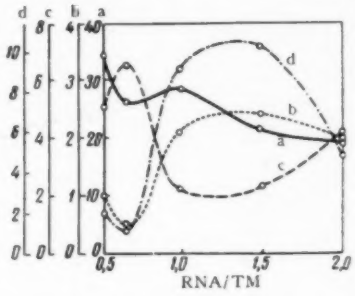


FIG. 15. RNA + TM complexes obtained at pH 4·6 and for various initial weight ratios of RNA and TM. Symbols as in Fig. 8.

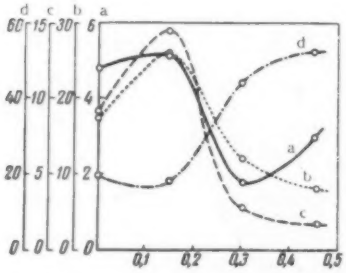


FIG. 16. Effect of NaCl concentration on complexing of RNA + OA. Symbols as in Fig. 8. Abscissa, molar concentration of NaCl in medium.

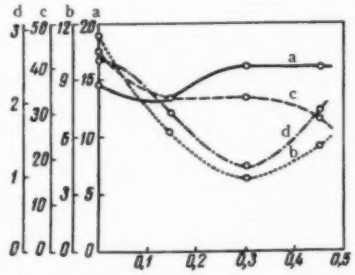


FIG. 17. Effect of NaCl concentration on complexing of DNA + TM. Symbols as in Fig. 8. Abscissa, molar concentration of NaCl in medium.

simultaneous disappearance of static paramagnetism. In other cases the intensity fell more slowly and the signal diminished by half after 1–2 months storage. *In vacuo*, the reduction in intensity occurred more slowly.

## CONCLUSIONS

The experiments with denaturation of DNA showed that the anomalous magnetic properties of nucleic acids that we detected are largely determined by the maintenance of an orderly native structure of the biopolymer. Both heat and acid denaturation led to reduction in the intensity of the signal. Obviously, the production of wide lines of magnetic resonance in this case, as in the case of synthetic polymers [7, 8], is associated with the existence of crystalline regions in which the planes of the individual

purine and pyrimidine bases form an almost linear orderly structure in the form of a column of parallel heterocycles. In the case of nucleic acids it is a matter of the regions of crystallinity within one macromolecule; in the case of synthetic polymers in the lattice of the entire solid samples. In the present article we shall not deal with the problem of the origin of these unpaired electrons.

The formation of a complex with protein, leading to a sharp increase in intensity of the wide line of magnetic resonance, apparently resises the number and possibly, the size of these intramolecular regions of crystallinity. The experimental data presented in this article indicate, as a whole, that increase in intensity of a signal of magnetic resonance on complexing is the result of structural changes and not the incorporation into the sample of an additional impurity. It should be noted that the formation of a complex with proteins leads to a change not only in the magnetic but also other properties of nucleic acids. Possibly the inhomogeneity of the original DNA preparation used in our experiments was due to the presence of small amount of nucleoproteins. Butler [14, 15] has refferred to the effect of small amounts of residual protein on the sedimentation properties of DNA.

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## SUMMARY

(1) An investigation has been made of the effect of heat and acid denaturation on the spectra of magnetic resonance of deoxyribonucleic acid. The intensity of the signal fell during denaturation.

(2) The effect of complexing with proteins on the magnetic properties of nucleic acids in various conditions has been studies. Complexing as a rule sharply raised the intensity of the magnetic resonance signals.

(3) The experiments carried out confirm the previous assumption that the anomalous magnetic properties of the nucleic acids and nucleoproteins are essentially determined by the structure of these compounds.

We wish to take this opportunity to express our thanks to Prof. A. G. Pasynskii for fruitful discussion on a number of problems touched upon in the present article.

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* There is a translation into English of this work.

VOL
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196

# STABILITY OF MEMORY OF THE BRAIN TREATED AS A SYSTEM OF AUTONOMIC "MEMORY"*

G. B. LINKOVSKII

Institute of Radiotechnics and Electronics, U.S.S.R. Academy of Sciences

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## 1. *Introduction*

THIS paper is a continuation of investigations the results of which were reported to the Session of the Commission for the Application of Mathematics in Biology of the Moscow Society of Naturalists attached to the Moscow State University on the theme "Brain as a system of autonomic memory" held at the end of 1957 [1]. Supplementary problems in relation to the interpretation of the memory of the human brain as systems of autonomic "memory" are first dealt with: then the physico-methematical characteristics of stability of memory are considered.

The supplementary problems include: (1) the scope of memory and training of memory; (2) quantizing of impulses arriving in the brain and the role of the sense organs; (3) the resolving power of the sense organs and its correspondence with the scope of the specialized memory; and (4) the peculiarities of musical memory, memory of taste and smell.

The physico-mathematical aspects of stability of memory are based on consideration of the iteration characteristic of an elementary non-linear autovibratory system, the concept of which derives from analysis of the transitional system of motion. Therefore, the exposition is given in the following order: (1) construction of the motion of the system for a given function of the initial impulse; (2) the transitional system to the inherent vibrations; and (3) resistance to disturbances of the amplitude.

A geometrical and analytical description is given and, in particular, reference is made to the analytical sign of stability to disturbances in amplitude. The concept of an ideal iteration characteristic is introduced with a discussion of the problem of the similarity of the iteration characteristic of the elementary auto-vibratory systems of the memory of the brain to an ideal iteration characteristic. It is pointed out that the instability of memory of the brain is associated with "damage" to the iteration characteristic of the elementary organs of memory for various reasons.

In the paper [1] a hypothesis (based on certain findings in a study of the brain structure) was put foward on the dynamic memory of the human brain, if not as a whole, then a number of its parts treated as a collection of non-linear autovibratory systems with feedback, so-called systems of autonomic "memory". A physico-mathematical theory of dynamic memory was given which demonstrates the existence of two systems

* Biofizika **5**: No. 6, 655–662, 1960.

of autonomic "memory": (1) systems of "absolute" memory; and (2) systems of "relative" memory. It was assumed that the brain possesses "relative" memory.

By virtue of the latter circumstance the brain memorizes only stopped impulses with a duration $\tau' < \tau$ where $\tau > 0$ is the "retardation" of the system. The motion of autovibratory non-linear systems of the "relative" memory is described by functional equations with a retarding argument of the form

$$x(t) = A[x(t)]x(t - \tau), \tag{1}$$

where $A\,[x(t)] = a_0 + a_1x(t) + a_2x^2(t) + \ldots\ldots a_nx^n(t)$ and $a_0, a_1, a_2 \ldots\ldots a_n$ are constants with $n$, an integer $> 1$.

If an impulse entering the brain has a duration of $\tau'$ greater than $\tau$ then the cells and nerves constituting one elementary vibratory system memorize the part of the impulse with a duration $\tau$, the other vibratory systems memorize $\tau' - \tau$ of the duration of the impulse. Hence, if we consider that the retardation $\tau$ in all elementary vibratory systems is the same, then in the memorizing of given impulse of duration $\tau'$ there takes part $[\tau'/\tau]$ elementary vibratory systems where [...] is the corresponding fraction of the whole part.

The scope of the memory of the brain depends on the number of elementary autovibratory systems and, of course, is quite restricted. However, for different individuals the scope of the human brain differs. Here, we consider it appropriate to point out that apparently, in the human brain at birth there is a certain store of incompletely formed autovibratory systems and therefore, training helps to complete conversion of these foci of memory into full valued aggregates of elementary systems of autonomic "memory", in line with the role of repetition indicated in [1]. Thus, man is born with rudiments of memory which, during his upbringing and life, are converted by differing degrees into full valued elementary organs of memory. Apparently, one of the modes of such training of memory is learning of verses and also foreign languages in childhood.

The problem of the stability of memory to disturbances in the brain itself is important. Before turning to this problem we would note that the impulse entering the brain is already stepped (without conversion of the time characteristic of the impulse). Naturally, the question arises as to which organs perform such quantizing of the impulses arriving at the brain, i.e. are responsible for their replacement by a stepped impulse. This question was not considered in [1]. The impulses are perceived by the sense organs: the auditory apparatus, the visual organs and obviously the nerve endings of the skin and also the organs of taste and smell. Like any instrument they have a restricted resolving power, i.e. can distinguish ordinates of the impulse differing from one another by not less than $\delta$. This value $\delta$ is called the resolving power of the instrument; it varies for different sense organs.

Of course, different individuals have different resolving powers. Obviously, in persons with musical hearing, the resolving power of the auditory apparatus is quite high (discrimination also occurs in terms of frequency).

Modelling of musical memory by frequency was not considered in [1]. It may be assumed that in the brain there are nerve cells—resonators which perceive the corres-

ponding frequencies. Since sound has amplitude and frequency and perception of both occurs simultaneously then the periodic reproduction of an impulse (amplitude) is accompanied by the periodic reproduction of the corresponding frequency present with the amplitude. Thus, we consider that the resonator cells reproduce the frequency only with period $T = \tau$.

It is obvious that gustatory sensations and olfaction are the result of perception of quite definite wawes emitted on vibratory movements of the corresponding molecules of the substances. Therefore, it may be expected that the memory of taste and smell is similar to musical memory—in the sense of the presence of resonators but of other frequencies.

Thus, we may speak of the resolving power of the resonator cells by frequency.

Professional tasters have a high resolving power of the gustatorynerves. Apparently, different regions of the elementary systems of memory in the brain correspond to these different organs. The greater the resolving power of these senses, the closer the systems of "relative" memory approximate to systems of "absolute" memory, i.e. the impulse is memorized with a greater number of steps.

In the animal world, for example, in dogs, there is an exceptionally high resolving power of the organs of smell with simultaneous development of corresponding "memory" in the brain. It is possible that this memory is also of the autonomic type indicated above. Such a development of a special form of memory is associated with the conditions of existence of the particular representative of the animal world and its progenitors (heredity occurs).

Since the receptors of the impulses from the external environment (sense organs) and the corresponding regions of memory in the brain form a single whole, a large volume of specialized memory and a high approximation of the elementary vibratory systems to systems of "absolute" memory, are matched by a high resolving power of the sense organs which directly perceive the impulses from the external environment. Therefore, the given individual is born not only with rudiments of memory but also with rudiments of resolving power of the sense organs. The latter, during training and life is also raised. Of course, the conditions of existence in the external environment, i.e. the external environment itself, have an effect on the development of the resolving power of the sense organs and of the organs of elementary memory in the brain.

## 2. *Iteration characteristic of elementary autovibratory system of "memory" and stability of memory*

Now we turn to the problem of the stability of memory to disturbances in the brain itself. It is necessary to clarify the problem of the equilibrium of the elementary non-linear autovibratory system, the motion of which is described by the functional equation (1). It is necessary that the iteration characteristic of the system (1) has a special form. The concept of iteration derives from an analysis of the transitional state of motion in these systems. We shall give a short outline of this.

*Construction of the motion of the system for a given function of the initial impulse.* Let us take the system (1). The function of the initial impulse $x(t) = \varphi(t)$ is given

for the initial segment of time with the length $\tau$ and consists of a group of rectangular impulses of different height and duration (Fig. 1). It is necessary to construct the motion of the system in the subsequent moments of time $t \geqslant t_0$. We shall show that this problem can be solved by the "piece" method. It is natural to apply such a method for an equation with constant retardation. The "piecing" derives from the fact that

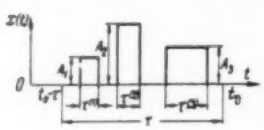

FIG. 1. Function of the initial impulse consisting of a group of rectangular impulses.

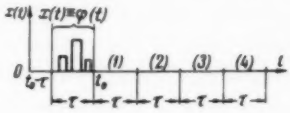

FIG. 2. Approach to construction of the motion of the system by the "piece" method $x(t) \equiv \gamma(t)$ —function of initial impulse.

initially, the motion of the system is constructed in the subsequent segment of time of length $\tau$. The motion obtained is taken as the initial function for constructing the motion of the system in the following segment of time $\tau$ and so on (Fig. 2). In the general case, for each segment of time $\tau$ we shall obtain a group of converted impulses which may be distinguished from each other in each interval of time $\tau$. Indeed, if an initial group of impulses enters the system, then in the subsequent intervals of time transformation of this group of impulses into others will occur. Our problem is that of constructing the converted impulses. We shall show that this construction of the motion of the system is a matter of a certain iteration process. For this purpose we turn to the functional equation (1). From it we can determine the function $x(t)$ in relation to $x(t - \tau)$:

$$x(t) = B[x(t - \tau)], \tag{2}$$

where $B$ is a certain function of the argument $x(t - \tau)$.

For function $B[x(t - \tau)]$ there may be two cases: (1) function $B$ is unique and (2) the function $B$ is multivalued. For the time being we shall confine ourselves to the first case. The relation (2) is the starting one for constructing the motion. Indeed, we shall write:

$$\bar{t} = t - \tau. \tag{3}$$

then

$$x(\bar{t} + \tau) = B[x(\bar{t})], \tag{4}$$

i.e. if the motion of the system at the moment of time $\bar{t}$ is known, then from the functional equation (4) we can find the motion of the system through and the time $\tau$. We have the motion of the system in the initial segment of time of length $\tau$. Then, using equation (4) we can construct the motion of the system in the following interval of time $\tau$. Each rectangular impulse will be converted into a rectangular impulse with the same duration $\tau(i)$, but the height of impulse in the general case will not remain the same as the previous one. We have:

$$A_i^{(1)} = B(A_i^{(0)}), \tag{5}$$

where $A_i^{(0)}$, the height of the initial impulse, $A_i^{(1)}$, the height of the converted impulse in the first interval of time $\tau$. Intervals between the individual impulses remain as before and $0 = B(0)$ the latter follows from (1). Thus, on conversion of a group of impulses only conversion of the amplitude occurs with the time characteristic remaining unchanged. Of course, this does not exclude the case where:

$$A_i^{(1)} = A_i^{(0)} \text{ for } i = (m), \tag{6}$$

i.e. from the series of values $i(i = 1, 2, 3, \ldots, l)$. In this case we have reproduction of the series of impulses in the first interval $\tau$. As will be clearly seen from the following remarks these values of the impulses will be repeated after the intervals of time $\tau$, i.e. will have a periodicity with a period $T = \tau$. If all the impulses in the initial group possess this property, then in the system, periodic motion of the initial group of impulses with a period $T = \tau$ will occur.

In the case of an arbitrary function of the initial impulse $x(t = \varphi)t$, by means of limiting transition in the division of this function into elementary rectangles, we find that these properties will also be true for it. Thus, it is possible to construct the motion of the system in the first interval of time $\tau$. We take the motion obtained as the initial one for constructing the motion of the system in the following interval of time. In fact, this may be done after taking into consideration the relation (4). Again, we have conversion of the amplitude characteristic of the first transformated group of impulses without change in the time characteristic of the impulses. Then we get:

$$A_i^{(2)} = B(A_i^{(1)}). \tag{7}$$

We shall denote by $n$, the number of the converted group of impulses and the height of the ith impulse in this group by $A_i^{(n)}$. Then

$$A_i^{(n)} = B(A_i^{(n-1)}), \quad \begin{pmatrix} n = 1, 2, 3, \ldots \\ i = 1, 2, 3, \ldots, l \end{pmatrix}. \tag{8}$$

Relation (8) reflects a certain iteration process. Construction of the motion of the system is a matter of an infinite series of iterations. It is not difficult to give a geometric interpretation of this iteration process. In turn, the geometric interpretation will subsequently help us to clarify the problem of the transitional state to the inherent vibrations and to indicate a way of explaining the stability of motion in the given non-linear system in relation to disturbances in amplitude of the impulse. In the main we shall make use of the geometric method of Lemeret which makes it possible to obtain a full qualitative picture of the relation between the values of the impulse $A_i^{(1)}$, $A_i^{(2)}$, $A_i^{(3)}$ ..... and the form of the function $B$ and the initial value of the implulse $A_i^{(0)}$. We shall call the function $B$ the iteration characteristic of the system. To construct the iteration characteristic we can also define the reciprocal function $B^{-1}$.

$$x(t - \tau) = B^{-1}[x(t)]; x(t - \tau) = \frac{x(t)}{A[x(t)]},$$

On the abscissa we shall plot $A^{(n-1)}$ and on the ordinates $A^{(n)}$ (Fig. 3). From the origin of coordinates we construct the bisector $A^{(n)} = A^{(n-1)}$ and plot the curve $A^{(n)} = B(A^{(n-1)})$. The method of defining $A^{(1)}$, $A^{(2)}$, $A^{(3)}$ ... from the given $A^{(0)}$ can be seen from the construction in Fig. 3. The details of graphic iteration are known [2–4]. Let us now take the second case in which the function $B$ is multivalued. Generally speaking the mathematically ambiguous solutions (4) are equally valid; therefore, in the physico-technical problems additional conditions must be fixed to ensure choice of a solution from a series of possible ones. Then, the problem becomes physically defined. Such conditions may be, for example, $x(t) > 0$ or conversion of the impulses in the system occurs to the nearest values from formula (4). Thus, using the "piece" method we can successively construct the motion of the system if the initial conditions are known.

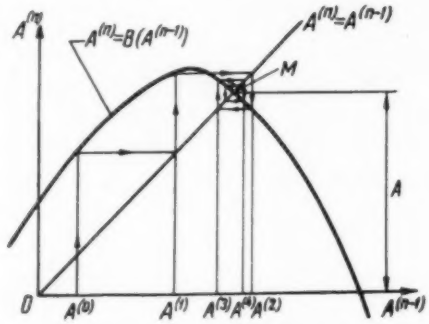


FIG. 3. Construction of motion of the system by means of the iteration characteristic (in particular, the case of asymptotic tendency to inherent vibrations is represented).

*Transitional regime for inherent vibrations.* Fig. 3 shows the case in which the iteration characteristic intersects the bisector at one point $M$. In general, bisection at several points is possible. If we have one initial impulse, i.e. the group of impulses consists of one rectangular impulse and is equal to the ordinate $A$ of the point of intersection $M$ (Fig. 3) then the system will periodically reproduce ("memorize") the same impulse. A similar phenomenon will occur also when in the initial group there are several such impulses. If the initial impulse $A^0 \neq A$, then the motion of the system may be constructed by the iteration method according to Fig. 3; but two solutions may be realized [2–4]:

(1) The infinite or finite succession of iterations meets the boundary at point $M$, i.e. after the transitional regime, a periodic motion of the impulse with height $A$ is set up in the system with a period $T = \tau$. The characteristics of the transitional state for these inherent vibrations can be seen from the iteration diagram. Such a case of the infinite sequence is depicted in Fig. 3. the time of the transitional state being infinite.

(2) The infinite succession of iterations does not meet the boundary at point $M$, i.e. in the system periodic motion is not set up.

Employing the accepted terminology, point $M$ is called the stationary point; in fact, point $M$ corresponds to a stationary system of vibrations. Likewise, we can have

several stationary points. Here, it is necessary to note the fact that from the work of Kennicks [2] it follows that the succession of iterations may meet the stationary point asymptotically if there is only one. If there are several such points then the

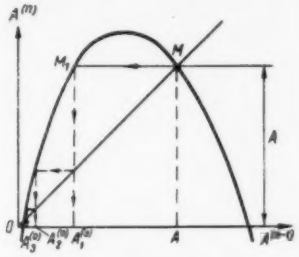


FIG. 4. Determination of the initial impulses corresponding to the finite transitional regime to inherent vibrations.

succession of iterations may meet them asymptotically (one set or another depending on the initial values of the impulses $A_i^{(0)}$). Hence, in the asymptotic sense:

$$A_i = B(A_i^{(\infty)}). \tag{9}$$

At the same time we can find certain values of the initial impulse $A^{(0)}$ which, after a sufficiently short time are converted into impulses of constant hight $A$, i.e. the transitional state to inherent vibrations will be sufficiently brief. Apart from the trivial case

$$A^{(0)} = A, \tag{10}$$

when the transitional regime is in general absent, it is possible to indicate simple means of distinguishing the particular values of the initial impulse $A^{(0)}$ possessing this property. For this purpose we turn to the iteration diagram (Fig. 4). Its use enables us

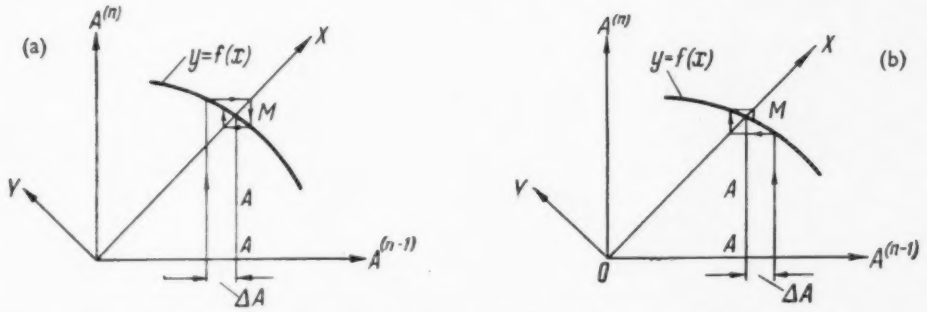


FIG. 5. (a) Case of stable "memorizing" of rectangular impulse for $f'(x) < 0$ (disturbance of amplitude to left); (b) Case of stable "memorizing" of rectangular impulse for $f'(x) > 0$ (disturbance of amplitude to right).

(as can be readily seen) to solve the problem in reverse. From point $M$ we draw a horizontal line to the intersection in $M_1$ on the characteristic. The abscissa of the point of intersection will be the closest value of the initial impulse $A^{(0)}$ obtainable by this

method. Next, from the point of intersection of the vertical line from $M_1$ with the bisector we draw the horizontal line to the intersection in $M_2$ with the characteristic to find $A_2^{(0)}$ and so on. Hence, we obtain the finite multiplicity of heights of initial impulse which, after a finite time (multiple $\tau$) are transformed into impulses of height $A$ which the system will then periodically reproduce. From this it is not difficult to determine the time $t$ of the transitional state: $t = m\tau$ where $m$ is the corresponding number of iterations.

*Stability to disturbances in amplitude.* It is of interest to elucidate the stability of the inherent vibrations for amplitude, namely, to disturbances in amplitude of a rectangular impulse. The stability of the inherent vibration of the system is explained in the following way. We shall give an elementary disturbance of the amplitude $\pm\Delta A$; if the succession of iterations agrees with amplitude $A$ the regime of inherent vibration ("memorizing") is stable (Fig. 5), otherwise it is unstable (Fig. 6).

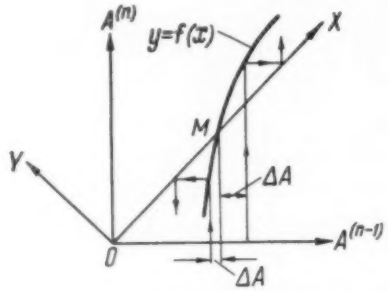


FIG. 6. Case of unstable "memorizing" of rectangular impulse for $f'(x) > 0$.

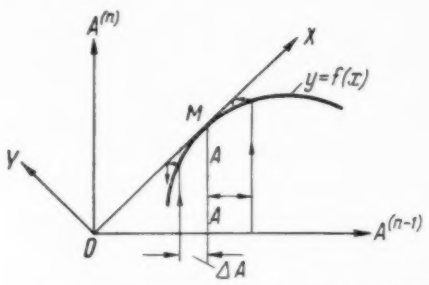


FIG. 7. Case of unstable "memorizing" of rectangular impulse for $f'(x) = 0$ (instability for $\Delta A > 0$).

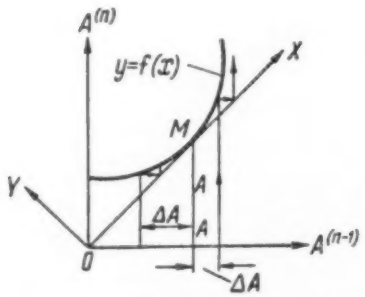


FIG. 8. Case of unstable "memorizing" of rectangular impulse for $f'(x) = 0$ (instability for $\Delta A < 0$).

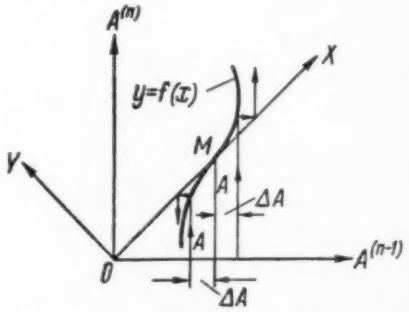


FIG. 9. Case of stable "memorizing" of rectangular impulse for $f''(x) = 0$.

We may use the following analysis sign. Let $y = f(x)$ be the equation of the iteration characteristic in the system of coordinates depicted in Figs. 5 and 6. Then, if at the corresponding stationary point the first derivative $f'(x) < 0$ the regime is stable, if $f'(x) > 0$ the regime is unstable.

Special cases arise if $f'(x) = 0$ (Figs. 7–10). In the case of Figs. 7 and 8 in which $f''(x) \neq 0$ we have an unstable regime. If the second derivative $f''(x) = 0$ and $f''(x)$ on transition through the stationary point from left to right changes from $+$ to $-$, we have a stable regime; on change of sign from $-$ to $+$ we have an unstable regime.

If the autovibratory system "memorizes" a multi-stepped impulse with $n$ heights of the steps of the impulse $A_i (i = 1, 2 \ldots n)$, then the iteration characteristic of the vibratory system $A^{(m)} = B(A^{(m-1)})$ must intersect the bisector $A^{(m)} = A^{(m-1)}$ at $n$ points and in such a way that the ordinates of the points of intersection are $A_i (i = 1, 2 \ldots n)$ (Fig. 11). Then the vibratory system will periodically reproduce the ordinates (levels) of the impulse (signal) $A_1, A_2 \ldots A_n$ with a period $T = \tau$. For these levels (differing from zero) to be "memorized" in a stable way the iteration characteristic $y = f(x)$ must have $(2n + 1)$ zeros with the necessary limitations for $f'(x)$.

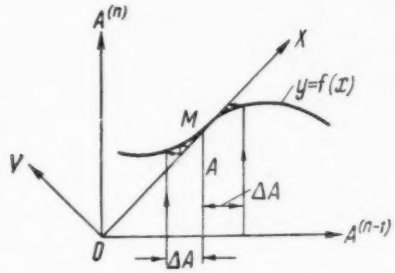


FIG. 10. Case of unstable "memorizing" of rectangular impulse for $f''(x) = 0$.

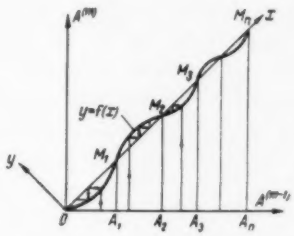


FIG. 11. General form of iteration characteristic on "memorizing" a rectangular impulse.

If the iteration characteristic has the form shown in Fig. 12 then it is sufficient to have $(n + 2)$ zeros of the function $f(x)$ to obtain $n$ stable levels of signal. We shall call the latter characteristic the ideal iteration characteristic.

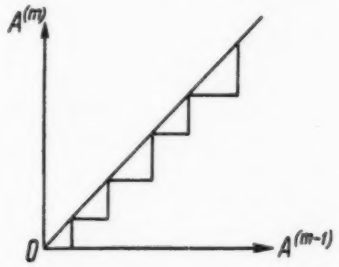


FIG. 12. Ideal iteration characteristic.

Now a word on the iteration characteristic of the elementary autovibratory systems of the memory of the brain. It is possible that it is not an ideal one but close to it, i.e. has few stable levels of the impulse delivered for memorizing. This is apparently to be explained by the fact that in nature living beings are very completely and economically built.

The observed fact on temporary or complete loss of memory are to be explained by cases of poor functioning of the elementary autovibratory systems of memory in particular, due to disturbance of their iteration characteristic because of various factors and the appearance of a large number of unstable levels. Obviously, in the life of the individual the elementary organs of memory also undergo certain evolution in connexion with the process of ageing of the body and the stability of memory of the brain eventually deteriorates. Of course, the physico-mathematical features of stability of memory of elementary systems of memory are accounted for by the quite definite structure of the nerve cells, nerves and other elements forming the elementary systems fo memory.

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* There is a translation into English of this work.

# PHOTODYNAMIC EFFECT OF VARIOUS FLUORESCENT DYES ON THE EXCITABLE GIANT AXON*

R. G. LYUDKOVSKAYA and L. P. KAYUSHIN

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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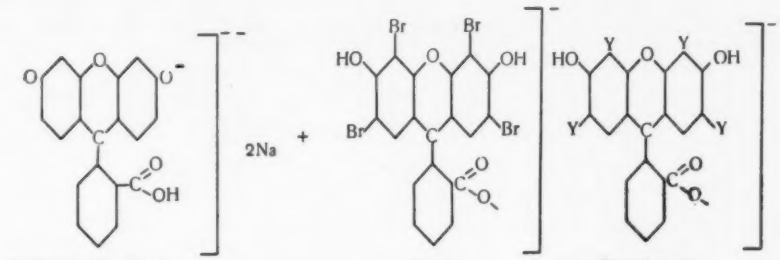
DYES are widely used in histochemistry and fluorescent microscopy to study localization and to establish the chemical nature of cell structures and the products of cell metabolism. Usually these methods are applied to sections of fixed or lyophilized tissues. Fluorescent dyes may also act locally on those structures and chemical substances of the living cell which selectively absorb dye. Visible light is used for this effect.

In conditions of adequate illumination most fluorescent dyes are toxic and lead to premature death of the living cell. This is a manifestation of the so-called photodynamic effect of dyes which, when excited by visible light, transmit the energy of photons to the substrate of the living system and in the presence of oxygen give photosensitized oxidization of the latter. Photodynamically active dyes lead ultimately to destruc-

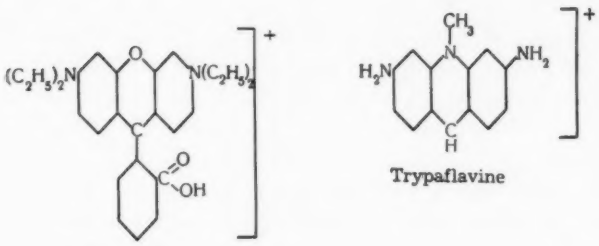
* Biofizika **5:** No. 6, 663–670, 1960.

tion of the protein and nucleoprotein substrate of the cell. However, in the first stages of the action of light, the dye may also be involved in metabolic processes concerned with transfer of surplus energy. In these stages the various dyes may act specifically on individual links of the metabolism.

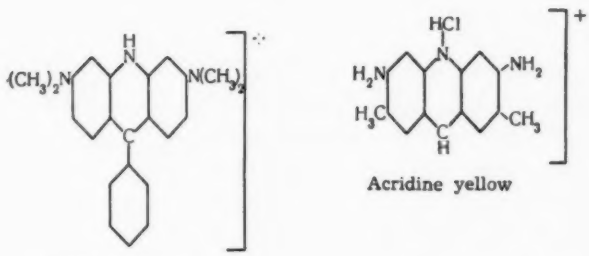
We used fluorescent dyes and light for selective treatment of certain groups of



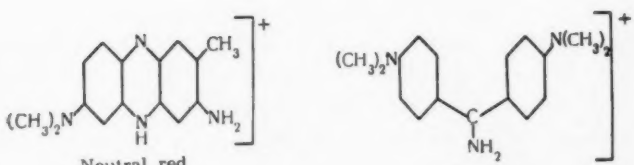

Sodium fluorescein (Uranine) Eosin Erythrosin



Rhodamine E Trypaflavine



Acridine orange Acridine yellow



Neutral red Auramine

substances and cell structures in order to elucidate their role in excitation of the living cell. Particularly suitable for this purpose is the giant nerve fibre of Cephalopoda constituting a comparatively simple protoplasmatic mass surrounded by a thin membrane and making possible continuous observation of the characteristics of the process of excitation and electrical activity.

We investigated the effect of a number of dyes of different structural formula, electric charge and intensity of fluorescence seeking to relate these properties to the ability of the dyes to bind selectively with definite substances and specifically to change the electrical activity of the axon.

The experiments described below were done on the 200–300 $\mu$ thick giant nerve fibres of *Sepia esculenta*. The action of fluorescein, eosin, erythrocin, rhodamine, trypaflavine, acridine orange and others on the electrical activity of the axon in the dark and in the light and the dependence of the photodynamic effect on the strength, duration and frequency of stimulation was investigated.

The carefully dissected axons were placed in a chamber with a glass bottom separated by transverse slots into sections filled with sea water. The light passing through the bottom of the chamber was focused on the stained area of the fibre (in one of the sections). The nerve was stimulated with $10^{-4}$ sec square pulses at a frequency of 1–50 c/s. The action currents were recorded by chlorinated silver electrodes immersed in the sea water, amplified and continuously observed on the ENO-1 oscillograph screen. The distance between the stimulating and recording electrodes was 10–15 mm but in many cases, for the purpose of precise determination of the stimulation threshold in the region under test, the axon was excited in the same place as the action potential was recorded, i.e. in the stained area of the fibre. The dye, diluted in sea water, was applied under one of the recording electrodes. The effect of the dye on the excitability and action potential of the axon was investigated first in the dark and then the dye was replaced by sea water and the stained area of the nerve about 1 cm long was illuminated. The experiments were carried out at a room temperature of 25–28°C.

We made a thorough study of the group of xanthine dyes (fluorescein, eosin, erythrocin, rhodamine B and 6G). Within this group comprising dyes of similar structure we sought to establish a relation between the photodynamic effect and the fluorescent properties of the dye and the sign of its electric charge.

*Dark effect of dyes of the xanthine group*

The dyes in low concentrations did not change the excitability of the axon in the dark, although they affected the value of the action current. The action current of the axon on staining with eosin (0·005–0·01 per cent) or erythrosin (0·01 per cent) rose after several minutes by 20–40 per cent. On staining with rhodamine the increase in the spike was less and was observed only in the first moments of application of the dye. Fluorescein in the dark did not change the value of the action current. A stimulatory effect of the dyes in the dark was detected only on fibres in a good functional state without the slightest trace of damage.

*Effect of visible light*

In the axon stained with eosin or erythrocin a prolonged potential developed in the light. This phenomenon has already been noted for the giant axon of the squid [1] under the action of light and eosin.

In all these cases the prolonged potential developed as a result of the extended duration of the descending phase of the spike. The duration of the action current increased in the case of eosin to 10–12 msec and erythrocin to 8–9 msec.

During continuous illumination of the axon, simultaneous with the retardation of the descending phase there was a reduction in the amplitude of the action potential and after several minutes (depending on concentration) the excitability vanished. Together with a change in the size and shape of the action potential on continuous illumination of the stained axon, periodic disappearance of the action current associated with suppression of the electrical reaction by light was observed.

The effect of eosin (tetrabromofluorescein) and erythrosin (tetraiodofluorescein) in the light was very similar and manifest at concentrations of 0·002–0·005 per cent.

Fluorescein (uranine) is very similar in its structure to eosin and erythrosin but, although having even stronger fluorescence, exerted almost no photodynamic effect and the stained axon on illumination for more than 30 min retained unchanged excitability and electrical activity.

Rhodamine 6G and B differ from the acidic negatively charged eosin and erythrosin ions in their basicity, i.e. have a positive charge and are excellent vital dyes. A concentration of 0·001 per cent proved effective in the light. The effect of the rhodamines B and 6G was similar. On illumination of the stained axon there was a slight rise in the duration of the action current (by a factor of 2–2·5) and its amplitude and shape was greatly changed. In the last stages of the photodynamic effect the threshold and lability of the axon also changed.

The shape of the action current (Fig. 1) diplayed a particularly curious change. The slope of the lower part of the leading edge of the spike gradually diminished whereas the slope of the peak region remained the same. At this point of transition there appeared a break and a plateau was gradually formed. The action current assumed a complex form with a distinct characteristic pre-spike potential. This potential, higher than the threshold stimulation, continued to exist alongside the action current and underwent changes in response to light. The duration of the pre-spike potential rose steadily reaching 3–5 msec and a value of up to 8 mV (rhodamine 6G). There was a simultaneous drop in the value of the action current and its origin was delayed.

After development of the plateau, the value of the pre-spike potential started to fall slightly with time but the rate of the rise (slope) of its leading edge was very constant and characteristic for dyes of this group. Thus, for rhodamine 6G the slope ($V$) of the pre-spike potential defined as the ratio of its amplitude ($A$) to the time of rise (to the plateau) $t$, was maintained within the limits 0·6–0·7; for rhodamine B, $V = 0{\cdot}7$-0·75.

Although a marked pre-spike potential appeared, no increase in threshold was

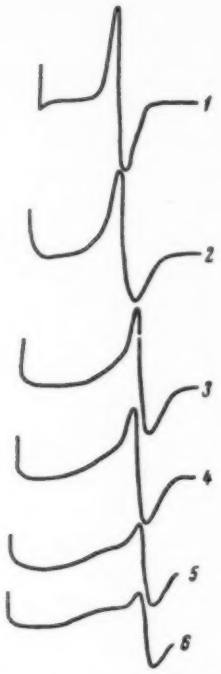



FIG. 1. Change in action potential on illumination of rhodamine stained giant axon of *Sepia esculenta* (rhodamine 6G 0·01 per cent).

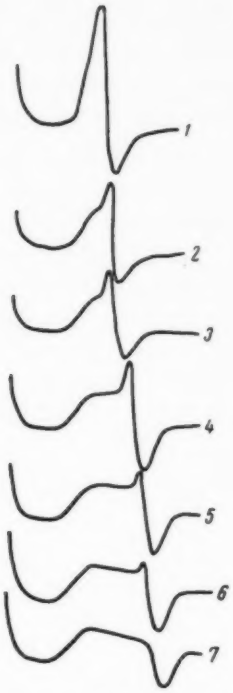



FIG. 2. Change in action potential on illumination of *Sepia* axon stained with acridine orange (0·01 per cent).

detected. The thereshold of stimulation measured in the stained and illuminated site either did not change in the first minutes or gradually diminished during 10–15 min, after which it started to display fluctuations, passing into a steady rise.

The effect of rhodamine as with eosin and erythrosin was accompanied by a reduction in lability and the frequency of stimulation of 30–40 c/s ceased to be completely reproducible.

*Photodynamic effect of dyes of the acridine group*

Of the dyes of this group acridine orange and yellow and trypaflavine were investigated. All three dyes are characterized by a positive charge of the ionized molecule in solution (see structural formula).

Trypaflavine and acridine yellow in concentrations up to 0·1 per cent in the dark exerted no detectable effect on excitability and the action current of the axon. In the light there was a slight increase in the duration of the spike after several miuntes. After washing with sea water the original parameters of the action current were restored.

Acridine orange in the dark in a concentration of 0·01 per cent caused in a number of cases, a slight reduction in the spike amplitude and an increase in its duration. On

switching on the light the characteristic changes in the leading edge of the action current began similar to those induced by rhodamine.

As the effect of light continued there was increasingly apparent, against the background of the spike, a pre-spike potential and a steady rise in its duration (Fig. 2). The value of the potential showed almost no subsequent change after reaching the maximum. The rate of rise ($V$), characterized by the ratio of the amplitude of the pre-spike potential ($A$) to the rise time $t$, also remained quite constant and was equal to 1·0–1·2 as against 0·6–0·7 for rhodamine.

Particularly characteristic of the pre-spike potential was the development of a protracted plateau which, as it extended, made the transition to a spreading process more difficult. The value of the pre-spike potential reached 2–5 mV and lasted 2–3 msec (in the conditions of external recording). It may be assumed that the real values of potential on intracellular recording are considerably greater both for rhodamine and for acridine orange, since the pre-spike potential reached 1/4–1/5th of the maximum value of the action current and on conversion to absolute values its true value should be 20–30 mV. It is interesting to note that a similar complex shape of the action current was formed in response to glycerol. After application to the area of the fibre of 20 per cent glycerol, on the leading edge of the spike there developed the same plateau and after a certain time the action current vanished. After washing with sea water the action current was restored for a short time but then the fibre died.

VOL. 5 1960

*Effect of neutral red*

Neutral red—a basic vital dye of the quinoneimine group was investigated in concentrations from 0·1 to 0·001 per cent. The latter concentration did not change the excitability nor the value of the action current of the axon either in the dark or in the light. Neutral red in a concentration of 0·1–0·01 per cent in the dark for 20–30 min and longer, did not change the action current. However, in the light, change in lability of the fibre was observed but shape and size of the action current remained unchanged.

The photodynamic effect of neutral red was investigated in relation to the frequency of stimulation. At a stimulation frequency of less than 30 c/s the stained nerve in the light retained excitability and conducting properties for a long time. Above 30 c/s the rhythm of stimulation ceased to be reproduced and periodic disappearance of the action current set in. This phenomenon was reversible and disappeared with reduction in the stimulation frequency. The photodynamic effect in this case displayed a clear relation to the stimulation frequency, the strength being unimportant. In the dark these phenomena were not observed.

*Effect of congo red (acidic fluorescent dye)*

Congo red in concentrations of 0·01–1 per cent did not exert a toxic effect on the excitable axon either in the dark or in the light. It should be noted that after staining with congo red the threshold of excitation and the action current become more stable in functionally unstable axons.

Auramine in normal conditions does not fluoresce. However, it is known that on fixation of the dye and formation of complexes it starts to fluoresce (fluorescence

is manifest on addition of nucleic acids). The dye proved to be very toxic, the threshold concentration being 0·0005 per cent. In toxic concentrations auramine depressed the excitability of the axon both in the dark and in the light. No distinctly marked photodynamic effect could be observed.

## DISCUSSION

The dyes of the xanthine group studied by us differed in intensity of fluorescence, the protein groups of the substrate affected and the electric charge on the ionized molecule.

The dye distinguished by the strongest fluorescence—fluorescein—exerted an insignificant photodynamic effect on the excitable axon. It is an interesting point that fluorescein, in contrast to eosin, does not affect the potential of the frog skin and the uptake of oxygen by the shin in the light [2]. Thus, no direct relationship was found between the intensity of fluorescence of the dye and its photodynamic effect.

Within this group of dyes, a characteristic aspect was the link between the electric charge on the molecule of the dye and the character of the photodynamic effect. In the light the acidic, negatively charged ions of eosin and erythrosin caused retardation of the descending part of the action potential. It is known that such dyes are absorbed on the surface of the cell forming complexes primarily with protein systems. Increase in the spike in the first stages of the action of the dye shows that the dye is incorporated into metabolic processes and briefly stimulates them. The ensuing reduction in the action current and the retardation in the descending phase are associated with depression and irreversible oxidation of enzymatic systems and destruction of the protein structure.

The basic dyes—rhodamine B and 6G belonging to the same group but having a basic character—penetrate the exoplasm and stain it well. They distinctively change the relation between the local and the spreading process. A pre-spike potential is distinctly evident next to the action potential although the observation was made at a distance of 10–15 mm from the point of stimulation. Acridine orange exerts an effect on electrical activity similar to rhodamine, the former being a basic dye belonging to the group of acridine dyes. In relation to the dyes of this group (diaminoacridine) it is established that they have an affinity for cell nucleoproteins and nucleic acids [4, 5] and may even serve to delineate DNA and RNA in the cells [6, 7]. It is known from the results of ultra-violet spectroscopy that the exoplasm contains nucleoproteins [7] and probably light acts on the complexes of nucleoprotein and dye.

Certain other factors are also important in determining the nature of the photodynamic effect. They must be taken into consideration in explaining the fact that the basic fluorescent dyes—trypaflavine and acridine yellow—did not exert an appreciable photodynamic effect on the axon. It must be assumed that in the formation of a complex of the basic dye and substrate, in all likelihood a ribonucleic substrate, fundamental importance attaches to definite side groups in the molecule of the dye and their affinity for the molecule of the substrate.

Rhodamine is characterized by the presence of two substituted amino groups

$N(C_2H_5)_2$ and acridine orange two $N(CH_3)_2$ groups. Trypaflavine and acridine yellow have two non-substituted terminal amino groups.

It is natural to assume that the specific photodynamic effect of the basic dyes on the electrical response of the excitable axon is determined by the presence of the $N(CH_3)_2$ and $N(C_2H_5)_2$ groups contributing to binding of the dyes and nucleoproteins.

A peculiar effect on the conducting properties of the axon is shown by the basic dye—neutral red. The photodynamic effect of this dye is mainly reflected in change in the lability of the nerve and depends on the frequency of stimulation and not on its strength. Using this dye as an example it is clear that the damaging effect of light is enhanced during functional activity of the nerve and has a direct bearing on the degree of excition of the substrate (frequency of stimulation).

However, the dependence of the photodynamic effect on excitation of the axon is part of the more general problem of interaction of processes of damage and excitation.

In the theory of Nasonov [8] it is assumed that both these phenomena have a common nature and are associated with denaturation phenomena in the protoplasm. From this point of view it must be expected that the damaging effect of certain other factors is also intensified by the functional activity of the cell. In fact, we previously reported that the damaging effect of ultra-violet radiation (2600–2800 Å) on a single nerve fibre is intensified on increasing the stimulating frequency [9] and after cessation of stimulation excitability is largely restored.

## SUMMARY

The effect of certain fluorescent dyes and visible light on the electrical activity of the giant axon of *Sepia esculenta* has been investigated.

The dyes of the xanthine group depending on the electrical charge on their ionized molecule exerted a different effect on the action potential of the illuminated axon. The acid dyes—eosin and erythrosin—caused a characteristic retardation in the descending phase of the spike; the effect of the basic dye—rhodamine—was associated with the appearance of a pre-spike potential of intensity greater than normal (8–9 mV) and of greater duration (5 msec) and depression of the spreading potential. Similar phenomena in the light were given by acridine orange—a basic dye of another group. This difference is due to the fact that the acid dyes are absorbed mainly on superficially located enzymes and other protein structures whereas the basic dyes—acridine orange and possibly rhodamine—are absorbed by the nucleoproteins of the exoplasm. A possible decisive factor in the formation of this complex is the presence in the dye molecule of two substituted amino groups. The different effect of light on the electrical activity of the axon in these cases points to the specific importance of the surface and intra-axon complexes in the formation of the different phases of the biopotential.

Besides light, an essential factor in bringing out the photodynamic effect of the dye is the activity of the living cell. The damaging effect of the dye and light is intensified at a high frequency of stimulation of the nerve and is not affected by the strength of the stimulation.

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VOL.
5
196

# PERCEPTION OF SOUND SEQUENCE*

L. A. CHISTOVICH

Pavlov Institute of Physiology, U.S.S.R. Academy of Sciences, Leningrad

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IT is known that human behaviour associated with sound signals may be very diverse. Man can, within certain limits, imitate sounds, carry out definite acts previously associated with sounds of a given character, memorize signals and compare them with others and finally, describe the properties of the sounds in words. It is obvious that all this diverse behaviour is possible only if as a result of the action of the sound an image of the signal is formed in the nervous system—a certain state reflecting (describing) the properties of the sonic action. Since measurement of the properties of sound signals (their spectra, fundamental frequency, periodic or non-periodic character, etc.) requires observation of the sound for a certain time it is natural to assume that the characteristics of the images must reflect the duration of the process at the input of the system. An essential experimental task is measurement of the duration of the process at the input corresponding to one image. In recent years attempts have been made to solve this problem by various methods [1–3]. The method used in the present work was to investigate the perception of sound sequence.

## 1. *Perception of sound sequence as one or two signals*

The object of the experiment was to determine the time interval between two brief tones for which they are perceived as two succeeding sounds.

*Methods.* Pairs of tones of 500 and 4000 c/s each wtih a duration of 20 msec were recorded on magnetic tape. Their duration and the interval between them were

* Biofizika **5:** No. 6, 671–676, 1960.

set by means of a two-channel electronic key. The sequence of the pairs was recorded at random both in respect of the interval of time between the deliveries and in respect of the succession of frequencies (500–4000 or 4000–500 c/s). Each pair was twice repeated in succession (with an interval of about 1 sec) then followed an interval of 5 sec during which the subject recorded his response (wether he heard one or two sounds) then the next pair was introduced.

The signals were divided into 2 series each containing 80 pairs (10 pairs for each of 8 intervals). There were 10 subjects, all of whom heard both series. Thus, 200 responses were obtained to each of the intervals between the deliveries. The results are given in Fig. 1 relating the frequency (percentage) of the response "2 sounds" to the interval of time between them. It will be seen that the percentage of replies "2 sounds" was very small at intervals shorter than 80 msec. Analysis of the material shows that the replies "2 sounds" obtained at intervals shorter than 80 msec were given in the main by only 2 subjects. These subjects accounted for 61 per cent of all these replies. It was concluded from questioning these subjects that they replied "2 sounds" because the signal perceived by them differed in character from each of the separate sounds and, consequently, was a dual one. Fig. 1 shows that an interval of 110–115 msec resulted in 50 per cent of the replies "2 sounds", which somewhat exceeds the interval of fusion of two sounds into one signal found by Urbantschitsch [4].

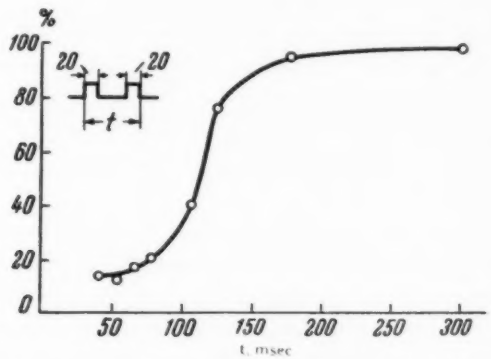


FIG. 1. Relation between percentage of replies "2 sounds" and interval of time ($t$) of tonal deliveries.

Thus, the results show that a sound sequence with an interval of time less than 80–100 msec is perceived as one signal and leads obviously to the formation of one sound image.

2. *Recognition of sequence of deliveries in time*

For short intervals of time between them two brief sounds are perceived as one signal and it is natural to expect that recognition of the sequence of the deliveries in time will be disturbed. To verify this experiments were carried out on hearing of the same signals as employed in the preceding experimental series; after hearing a pair

of deliveries the subject had to indicate which of them—high or low—was the first in the pair. Twelve subjects took part in the experiments; 500 replies were obtained for each interval.

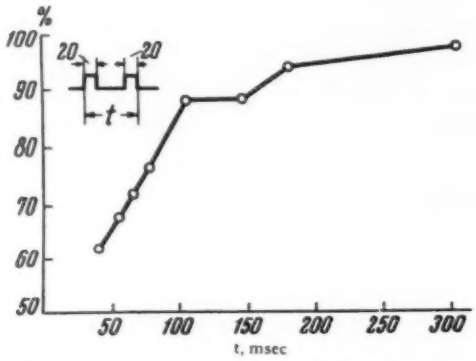


FIG. 2. Relation between percentage of correct recognitions of the frequency of the first in a pair of tonal deliveries and interval of time ($t$) of deliveries.

Results are given in Fig. 2 relating the percentage of correct replies to the interval of time of the tones. The complex form of the curve in Fig. 2 strongly suggests that disturbance in recognition occurs under the influence of at least two factors, one of which operates in the region of short intervals (up to 100 msec) and the other for average intervals. This is also indicated by the results from questioning the subjects. The subjects said that for average intervals they distinctly heard two sounds but did not, however, have time to "recognize" them. For short intervals the subjects heard one complex sound and attempted a guess from its character as to whether it began with a high or low delivery.

Thus, the results show that infallible recognition of the sequence of the deliveries requires very long intervals (up to 300 msec). However, recognition is to some extent also possible for very short intervals when both deliveries are perceived as one signal. This suggests that conversion of two deliveries into a perceptible signal (one image) is not accompanied by complete loss of information of the sequence of the tones in time. It is obvious that the problem of the reflexion in the properties of the images of the time characteristics of the signals is an extremely important one. Unfortunately, the work carried out in this direction is very scanty [5–7] and still does not allow any definite conclusions.

3. *Discrimination of the frequencies of tones forming sound sequences*

If two rapidly succeeding sounds lead to the formation of one image, it is natural to assume that the timbre of the image will be determined by the spectra of both sounds taken together. Then it may be expected that if a subject is presented with two sequences consisting of tones of different frequencies ($A_1$ and $A_2$) and identical complex sounds with a wide spectrum (B) then for short time intervals between A and B discrimination of these sound sequences will be difficult (total spectrum $A_1$ and B will differ little

from the total spectrum $A_2$ and B). With an increase in the interval the contribution of the spectrum of the complex sound to the image will gradually diminish and finally, a moment should be reached when the image will be determined solely by the tonal delivery and a further increase in the interval no longer improve discrimination. To test this assumption experiments were carried out on discrimination of two sequences.

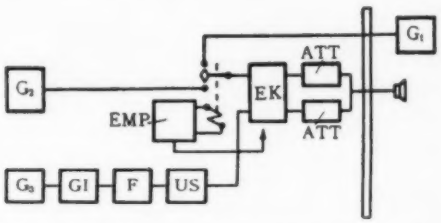


FIG. 3. Block diagram of experimental arrangement $G_1$ and $G_2$, generators of sound frequencies (EG2) used to obtain tonal deliveries; $G_3$, GI, F and US, arrangement used to obtain complex sound; $G_3$, generator setting the frequency of pulse sequence; GI, generator of the pulses; F, filter with band pass of 1500–3000 c/s; US, amplifier; EK, two-channel electronic key fixing duration of signals (tonal delivery and complex sound) and lag of start of complex sound behind start of tonal delivery; EMP, electromechanical switch starting electronic key at set intervals of time and effecting change-over of input of electronic key; ATT, attenuators.

*Methods.* The signals were obtained by means of the arrangement depicted in Fig. 3. The frequency of tone $A_2$ (generator $G_2$) was kept constant during measurement. The frequency of the tone of $A_1$ (generator $G_1$) was varied by the subject. The complex sound B was a harmonic of 200 c/s in the 1500 to 3000 c/s band. The duration of the tonal delivery was 30 msec; the duration of the complex sound 100 msec, the intensity of the tonal delivery was about 40 dB above threshold and that of the complex sound 60 dB above threshold. In order to avoid training (which was particularly important in the fourth series of experiments, see below) the frequency of the tone $A_2$ was changed from measurement to measurement (within the limits of 1500–300 c/s). The subject changed the frequency of the tone $A_1$ until the sequences were perceived as uniform. The error in frequency adjustment (deviation of frequency established by the subject from the frequency of tone $A_2$) was expressed in the form of $\Delta f/f$.

The mean square frequency error, taken as the threshold for a given interval, was computed from 48 values of error in frequency adjustment (12 values for each of 4 subjects); the interval $t$ was defined as the distance in time from the start of the tonal delivery to the start of the complex sound.

The results are shown in Fig. 4 from which it will be seen that the threshold diminishes with an increase in interval, reaching a minimum at about 120 msec when it again rises somewhat. It is essential that the threshold does not reach the values observed on comparing two tonal deliveries acting in insolation in the absence of complex sounds (broken line in Fig. 4). The subjects stated that not only the character of perception but the method of comparison changed with change in the interval. For the shortest intervals they compared one with the character of the start of the other complex sound,

for average intervals they perceived the sequences (type of syllable "ie") and compared them with each other. For the longest intervals they perceived the tonal delivery and the complex sound as completely independent signals and now compared the tonal deliveries themselves as such. The complex sound wedged in between two deliveries (the complex sound of the first sequence) hindered recall of the first delivery.

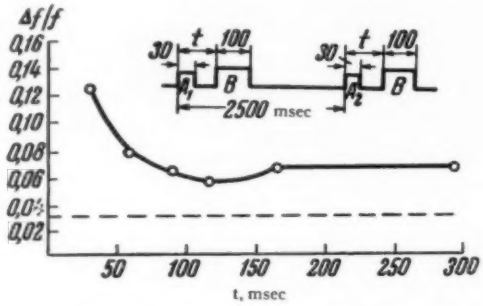


FIG. 4. Threshold of discrimination of frequency of tonal delivery in relation to interval of time between start of tonal delivery and start of complex sound.

From the description of the impressions of the subjects given above it was to be expected that replacement of the first sequence by one tonal delivery (exclusion of complex sound) should lead to reduction in the threshold for long intervals and to a rise in threshold for short intervals. In order to check this the following series of experiments was carried out·

4. *Comparison of frequency of an isolated tone with frequency of tonal delivery incorporated in a sound sequence*

The experiments were carried out with the signals schematically depicted in Fig. 5. Except for replacement of the first sequence by an isolated tone the experiments of the fourth series were completely identical with those of the third. The same 4 subjects took part in the experiments and the number of measurements was the same.

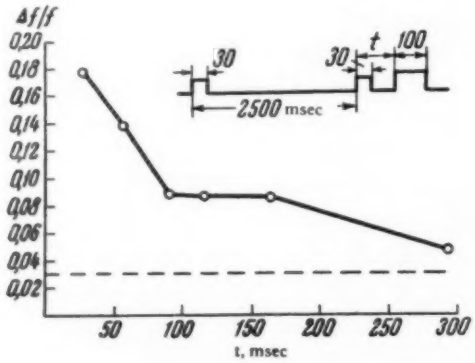


FIG. 5. Threshold of discrimination of frequency of tonal delivery in relation to interval of time between start of tonal delivery and start of complex sound.

The results are shown in Fig. 5. They indicate that a complex sound following a second tone influences the discrimination of frequency over a very wide range of intervals (up to 300 msec) in a complex manner. In the region of short intervals (up to about 90 msec) the thresholds progressively rise with reduction in the interval, in the region of average intervals (90–160 msec) the influence of the complex sound is still very considerable and does not depend upon the length of the interval. It will be seen that this pattern of influence is very similar to that which was obtained in the second series of experiments.

It is obvious that impairment of discrimination in the region of low intervals must be attributed to the fact that the second tonal delivery and the complex sound were perceived together, leading to the formation of a common image. This is in complete agreement with the findings of the first and third experimental series.

The problem of the impairment of discrimination in the range of average intervals is more complex. For these intervals man should already separately perceive the tonal deliveries (image should be defined already by its inherent properties). This is suggested both by the findings of the first and third series of experiments and also by the fact that man may correctly repeat (imitate) sounds with latent periods of less than 150 msec [3]. The impressions of the subjects for the average intervals are of interest. Subjects stated that they heard the first delivery very well but did not have time to "recognize" or "understand" it. It will be seen that this explanation coincides with that given by the subjects in the experiments of the second series. It is still not clear what "understand" and "recognize" signify. However, there is no doubt that for the subject to be able to decide on the identity of the second delivery with the first, or on their difference, he must not only perceive the second delivery (form its image) but make a comparison of the image of the second delivery with that of the first retained in his memory. It is natural to suppose that the process of comparison requires time. Thus, it is known that the latent periods of the reactions based on comparison or classification of sounds [8, 9] are much greater than the latent periods of the reactions of imitation [3].

We now turn to comparison of the findings of the third and fourth series of experiments, (see Figs. 4 and 5). The fact that for short intervals the thresholds were higher in the fourth series is quite understandable. The image elicited by the tonal delivery and the complex sound must have very little in common with that elicited by a tonal delivery alone and comparison of them naturally must be more difficult than comparison of two essentially similar images (third series of experiments). It is also understandable that for long intervals the results were better in the fourth series. Exclusion of a complex sound wedged between two tonal deliveries naturally must have facilitated retention in the memory of the image of the first delivery.

The difference in the thresholds of the third and fourth series of experiments for average intervals is most interesting. Had the subjects compared only the tonal deliveries in both series, then the thresholds determined in the third series ought to have been higher than those of the fourth series of experiments.

In fact, the reverse was observed. This is understandable if it is assumed that on comparison the subjects may use not only the images of the elements of sequence but

also information on change in sound on transition from one element of the sequence to another (images of sequences).

By analogy with perception of thesounds of speech we would note that the experiments of the fourth series correspond to that case in which man must compare an isolated sound of speech (for example, "i") with a similar sound forming part of a syllable ("ie"). Naturally, in this comparison one can base oneself only on the absolute characteristics of the sound. The experiments of the third series correspond to the case in which two syllables—with two uniform sounds in the syllable—have to be compared. In this case it is possible to utilize both the absolute characteristics of the first sound and the difference between the first and second sounds of the syllable (character of change of sounds in the syllable).

## CONCLUSIONS

The findings obtained in all four series of experiments suggest the conclusion that sequences of two signals in a time lapse of less than 100 msec lead to the formation of an overall sound image reflecting the properties of both signals together. The findings of the second series indicate that the images corresponding to short sequences, varying only in order of sequence of signals in time, differ from each other. This suggests that the information on the sound contained in the image also includes certain information on the time course of the signal. It is still not at all clear how this time aspect of the signal is measured and how it agrees with the phenomena of averaging of the signalling time as indicated both by the findings of the third and fourth series of experiments in the present paper and by the numerous findings obtained in other investigations [10–14].

If the second signal of the sound sequence lags behind the first by an interval of time greater than 100 msec then the sound sequence is reproduced as a sequence of images. The first image in time is determined by the properties solely of the first signal and does not depend on the properties of the second. It is important, however, that perception of the first signal (formation of image determined by the properties solely of the first signal) is still not a sufficient condition for its recognition—deciding on the signal (classification of it as high or low, coinciding with the memorized signal or differing from it). The process of recognition takes time and it may be disturbed by the effect of the second signal of the sequence acting 150–200 msec after the start of the first. It may be assumed that the cases of disturbance in detection or discrimination of signals by stimulations at time intervals greater than 100 msec after the start of the signal [15–17] must be attributed to disturbance in the process of recognition.

It is clear that further investigation of perception of sound sequences is of considerable interest, since it makes possible experimental differentation of the various stages of treatment by the human nervous system of the data on signalling.

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960

# EFFECT OF VERY INTENSE OPTICAL STIMULATION ON THE VISUAL, AUDITORY AND SKIN ANALYSERS OF MAN*

P. O. MAKAROV

Zhdanov State University, Leningrad

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THE theory of stimulation–excitability has been developed not only by physiologists but also by biophysicists [1–2]. Excitability is one of the basic properties of living organisms; it is usually determined by the lowest threshold strength of stimulation. But in natural conditions excitable systems and natural receptors—sense organs—receive stimulations varying greatly in strength. With modern techniques light stimulations can exceed threshold ones some thousand million times.

According to Nutting [10] the upper threshold—the threshold of blinding sensation—is determined for a visual field of 3–4° by the relation:

$$g = 8\sqrt[3]{B},$$

where $g$ is the intensity of the binding stimulation and $B$ is the intensity of the initial background light stimulation of the dark-adapted eye.

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† Read on 24 February 1959 to the Scientific Session of the Leningrad State University.

The range $D$ of light excitability of the dark-adapted human eye is $D = M/m$, where $M$ is the intensity of the upper threshold of the blinding stimulation and $m$ is the threshold of light excitability of the dark-adapted human eye ($D$ is of the order of $10^{11}$). Of the same order is the range of excitability of the human auditory analyser.

Measurement of the range of an isolated motorneurone [11] has revealed six steps (ranges) in its reaction to short, continuously increasing electrical stimulations: (1) pre-exci-tation, (2) excitation, (3) multiplication, (4) depression, (5) restorable, and (6) independently non-restorable parabiosis (*sic*). The refractory period i.e. complete restoration of excitability after the stimulus, is greater the stronger the stimulation. While for near-threshold stimulation of the neuromotor unit of vertebrates it is 1–3 msec and for the threshold 30–50 msec, for the parabiotizing stimulus it reaches 36,000 msec and more, i.e. more than 36 sec.

The object of the present investigation was to measure the refractory period in the human visual system due to brief very interse stimulation (within a range of thousands of millions of rheobases) by light from impulse gas discharge lamps. Measurement was made in man of the refractory period in: (a) the illuminated eye; (b) the non-illuminated eye; (c) in the skin analyser; and in (d) the auditory analyser.

## METHODS

The fixed optical adequatometer [12] was used to deliver to a definite region of the retina an optical stimulus graded in strength, duration and area (Fig. 1). Platinum contacts with micrometer screws ensured presentation of an additional skin or sound stimulus with any micro interval before or after the optic stimulus. An ISS-250 impulse lamp with an intensity of illumination of $4 \times 10^5$ cd was inserted into the platinum contacts. The pupil of the observer received illumination of $2 \times 10^5$ lx. The duration of the flash of the ISS-250 impulse lamp used was determined from the formula:

$$t = \frac{0{\cdot}01\, l\, C}{S}$$

where $l$, the length of the lamp column filled with inert gas, $C$, the capacity of the condenser, $S$, the area of the cross section of the lamp column. Calculations and checking showed that for the operating conditions of the lamp used the duration of the flash was 0·0009 sec (0·9 msec).

The arrangement for incorporating the ISS-250 lamp (IL) into the contactor $K$ of the optic adequatometer is shown in the left side of Fig. 2. The pendulum swings and through contact $K$ switches on the ISS-250 lamp which instantaneously flashes and illuminates one or both eyes. After this brief very intense flash the pendulum travelling further, after a set interval of time (from 0·5 to 500 msec), gives a weak light flash the threshold intensity of which we measure and from it assess the effect of the short very intense illumination on the excitability of the eye just illuminated or (in the second series of observations) of the other unilluminated eye.

Preliminary 30-min dark adaptation was carried out. By means of a red fixation

point located at an angle of 8° from the fovea centralis a definite region was stimulated with very intense or threshold light. The size of the inspection disk was 4 or 7°.

In the second series of observations one eye was illuminated with a super bright short flash, the other, left in the dark and subjected only to threshold illumination, was tested for change of excitability. A special hood was slipped on to the ISS lamp and the eye to be illuminated for this purpose. In the other series of experiments the brief

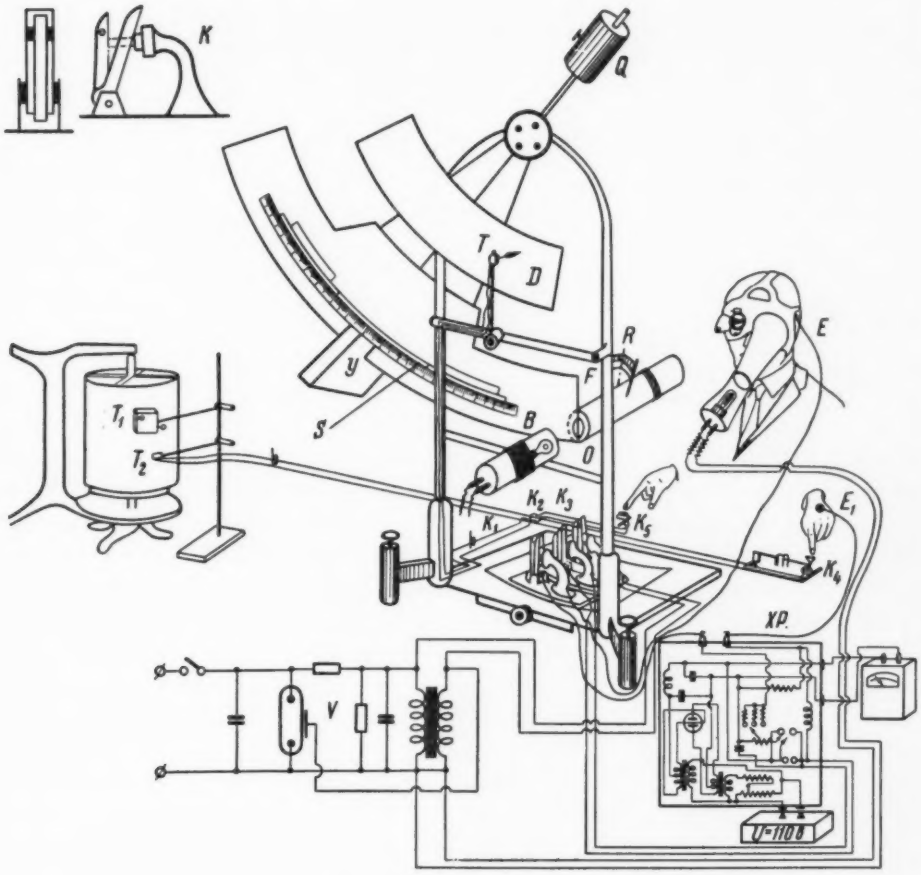


FIG. 1. Optical adequatometer. *B*, illuminator; *O*, adaptometer; *F*, part of adaptometer for neutral or coloured light filters; *R*, small arm, moving along a scale controlling opening of iris diaphragm; *S*, slit moving to and fro with bob of pendulum; *y*, projection for making contact; *D*, recording disk; *Q*, counterpoise regulating velocity of swing of pendulum; *K*, $K_1$, $K_2$, $K_3$, moving triple pole contacts; *T*, electric time marker; $T_1$, $T_2$, electrical markers of subject's responses; *V*, ISS-250 impulse lamp; *E*, $E_1$, active and indifferent electrodes respectively (*sic*); XP Burginon type chronaxometer $K_4$, $K_5$ keys.

very intense optical stimulation was combined at a definite micro-interval with auditory or electroskin stimulation which made it possible to measure the changes in excitability in the other analysers of the subject in response to very intense optical stimulation.

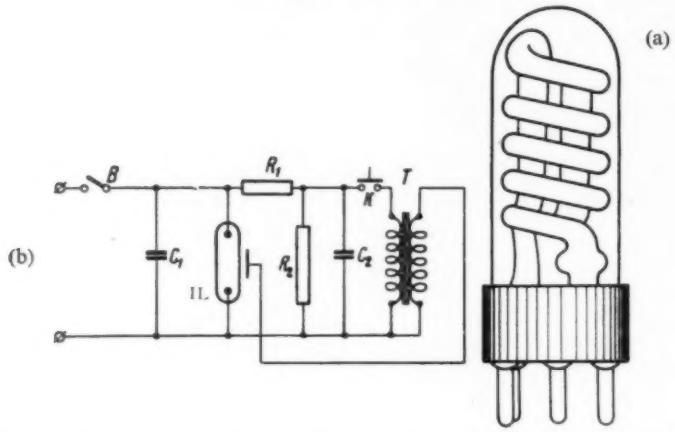


FIG. 2. ISS-250 impulse lamp (a). On left (b) diagram of its inclusion into platinum contact $K$ of adequatometer. $T$, transformer; $C_1$ and $C_2$, condensers; $R_1$ and $R_2$, resistances; IL, impulse lamp; $B$, key.

## RESULTS

The investigations were carried out on three normal observers aged 40–50 years; 2 females and 1 male. In the first series of investigations the observer was adapted for 30–40 min to the dark until a relatively constant background of sensitivity $E$ of the visual analyser was established; $E = 1/it$ where $i$ is the threshold intensity of light stimulation and $t$ is the time. Then, a brief very intense light stimulus was delivered with an intensity of 400,000 lx and duration 0·9 msec and at various intervals after it the thresholds of light excitability were determined. Table 1 and Fig. 3 give typical results.

TABLE 1

| Ttime of observation | Interval after very intense stimulation (msec) | Sensitivity as % of original | Time of observation | Interval after very intense stimulation, (msec) | Sensitivity as % of original |
|---|---|---|---|---|---|
| 10 hr 15 min | 5 | 6 | 10 hr 37 min | 250 | 84 |
| 10 hr 20 min | 30 | 27 | 10 hr 40 min | 280 | 85 |
| 10 hr 23 min | 80 | 33 | 10 hr 44 min | 330 | 90 |
| 10 hr 26 min | 105 | 50 | 10 hr 50 min | 10* | 32* |
| 10 hr 29 min | 120 | 66 | 10 hr 52 min | 35* | 44* |
| 10 hr 32 min | 150 | 71 | 10 hr 55 min | 60* | 65* |
| 10 hr 35 min | 180 | 82 | 10 hr 59 min | 110* | 95* |

Note: Sensitivity ($E$) was determined from formula, $E = 1/it$.
*Threshold stimulus precedes super-maximal one.

The table presenting the data for one subject and Fig. 3 (the data for another subject) show that brief very intense light stimulation causes a sharp drop in the light sensitivity investigated by us for up to 330 msec. For the longer intervals, 0·5–5 sec, investigated for electrosensitivity of the eye by Motokawa [13] and Korzum [14] the sensitivity was found to increase and not decrease. It is necessary to take into account the differences in our

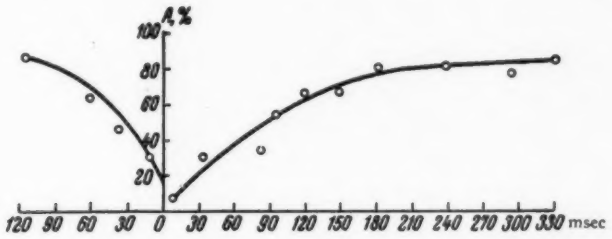


FIG. 3. Change in light excitability ($A$) as percentage of original under influence of a very intense optical stimulus. Abscissa on right, intervals in msec after very intense optical stimulus; on left, before very intense optical stimulus.

technique from that of Motokawa. We determined the changes in sensitivity to adequate light stimulations caused by a very intense light stimulation in micro-intervals of time (msec), whereas Motokawa and his colleague and also Riggs and Korzum determined the changes in electrosensitivity in macro-intervals (sec).

It should be noted that long before Motokawa, in 1939, we investigated the shifts in electrosensitivity of the human visual analyser occurring in response both to light and to electrical stimulation of the eye within a range of micro- and macro-intervals of time and obtained biphasic changes; in the first phase reduction in sensitivity, in the second a rise. Obviously, very intense light stimulation causes biphasic changes in sensitivity of vision; initially sharp depression then a rise. What is the origin of these changes in excitability caused by brief very intense light stimulation? What is the role of the peripheral (retina) and the central links of the visual analyser in this phenomenon?

To answer these questions we carried out further investigations. In one series, the brief very intense stimulation acted only on one eye and the changes in light excitability and electroexcitability induced by it after various micro- and macro-intervals were measured on the other non-illuminated eye. It is clear that the changes in electro-excitability and light excitability of the non-illuminated eye must depend on the changes in the central links of the analyser. Investigations showed that brief very intense light stimulation causes changes in excitability not only in the visual but also in the auditory analyser (to a lesser degree). In this series of experiments at various intervals of time after very intense light stimulation, a tone from a sound generator was switched on for 100 msec by means of the contacts of the adequatometer. Changes in excitability of the auditory analyser in response to very intense light stimulation were compared in the same experiment with those in light excitability and electro-excitability of the other non-illuminated eye. The results of this series of experiments are presented in Table 2 and Fig. 4.

Table 2 and Fig. 4 illustrate two typical observations on two subjects. The fall in excitability of the human visual and auditory analysers under the influence of very intense monocular light stimulation is greater the shorter the interval from the start of light stimulation. With an increase in the interval between the very intense stimulation and the test stimulation the fall in light- and electro-excitability of the eye and

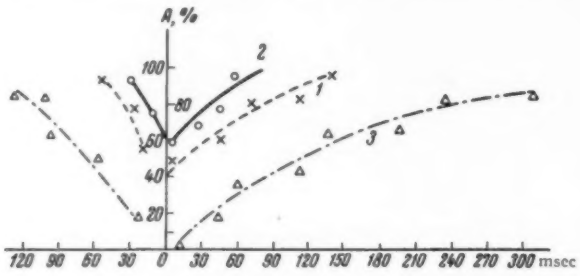


FIG. 4. Change in electro-excitability of eye "phosphene" (1); auditory excitability "hearing" (2) and optical stimulus (3). Significance as in Fig. 3.

also the auditory excitability diminishes. The first phase in the shifts of excitability generated by the brief very intense light stimulation terminates after intervals of 300–400 msec for light excitability, 150–200 msec for electro-excitability and 70–100 msec for excitability of the auditory analyser.

TABLE 2

| Time | Interval after very intense light stimulation of left eye (msec) | Changes in light excitability of right eye as % of original | Changes in electro-excitability of right eye, % | Changes in excitability of auditory analyser as % of original |
|---|---|---|---|---|
| 10 hr 11 min | 4 | 3 | — | — |
| 10 hr 13 min | 3 | — | 38 | 52 |
| 10 hr 16 min | 17 | — | — | 60 |
| 10 hr 18 min | 45 | 18 | 51 | 69 |
| 10 hr 20 min | 65 | 30 | — | 90 |
| 10 hr 23 min | 70 | | 75 | — |
| 10 hr 25 min | 105 | 40 | 89 | — |
| 10 hr 28 min | 130 | 67 | 96 | — |
| 10 hr 30 min | 190 | 70 | — | — |
| 10 hr 33 min | 230 | 82 | — | — |
| 10 hr 35 min | 300 | 97 | — | — |
| | Super-maximum light stimulation following test one | | | |
| 10 hr 40 min | 9 | 17 | 50 | 72 |
| 10 hr 43 min | 18 | — | 70 | 94 |
| 10 hr 46 min | 30 | — | — | — |
| 10 hr 49 min | 58 | 49 | 92 | — |
| 10 hr 52 min | 100 | 80 | — | — |
| 10 hr 55 min | 120 | 95 | — | — |

Here, as in the preceding series of experiments, it was found that if the test stimulation over a certain interval preceded the very intense one (see lower part of Table 2 and left part of Fig. 4) then in this case the very intense light stimulation for a certain interval caused a fall in excitability both of the visual and auditory analysers of the subject. The effect of "back to front" stimuli has previously been studied by me [11, 12]. I assume that sensation is a phase process and at a certain stage of its maturation one can influence it either by attenuating it as in the present case, or by intensifying it. The maturing sensation then ceases to be vulnerable.

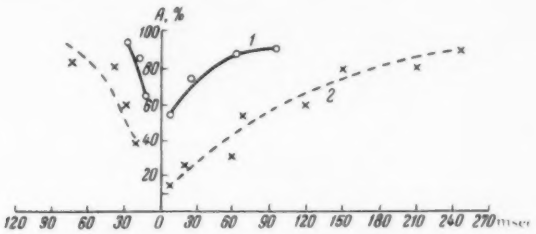


FIG. 5. Change in electro-skin sensitivity (1) under influence of brief very intense stimulus and sensitivity of other non-illuminated eye (2). Significance as in Fig. 3.

Fig. 5 shows a typical example indicating that the brief very intense light stimulation, in this case of the left eye, produces, as in the preceding series of observations, a sharp drop in the light excitability of the non-illuminated right eye. As the interval between the very intense light stimulation and the test illumination of the other eye was reduced and after intervals of 270–350 msec, the first phase of sharp decline in light excitability terminated. It is interesting that very intense light stimulation applied 90–100 msec after the test one, as already found in the previous experiments, also caused a reduction in light excitability.

An important aspect is that the fall in excitability due to very intense light stimulation for a certain time (up to 100 msec) spread to other analysers, for example, the skin analyser (Fig. 5). The point electrode (cathode) was affixed to the lower part of the right forearm at Erb's point of the ulnar nerve. The indifferent silver electrode (anode) a plate 100 cm² in area moistened with saline was attached to the left upper arm. The initial thresholds for the condensor discharges (with a capacitance of 0·1 $\mu$F) were rirst established. Then, after a chosen interval (for example, 10–30 msec and so on) after a brief very intense illumination of the left eye, the threshold of the electro-skin excitability of the right arm was determined. The threshold was up to 100 msec after brief very intense illumination as against 30 msec before illumination. Consequently, brief very intense light stimulation of one eye causes during intervals of 300–400 msec a sharp drop in the light- and electro-excitability of the other eye and the fall in excitability is detected not only in the non-illuminated eye but in the other analysers–auditory and skin.

Thus, brief very intense light stimulation in the form of a flash from an impulse lamp with an intensity of 200,000 lx causes a peculiar complex adjusting reflex, manifest in change in excitability both of the stimulated analyser itself and the other analysers.

This change is biphasic. Initially, there is a phase of sharp depression of excitability, then a phase of increase. The second phase developing in macro-intervals of time, i.e. after 1, 3 and 5 sec has been investigated by Motokawa and colleagues [13] and also by Riggs and Korzun [14] and others.

An essential point is that the shift is maximal for simultaneous presentation of the very intense and test stimulations; as the interval is increased, the shift diminishes. This is a comparatively rapid process of readjustment of the functional state of the analyser. Also it decreases with repetition.

If the test stimulus is presented at a definite interval before the very intense one, then a change in the refractory period of up to 100–150 msec is also observed. It is difficult to represent the phenomenon of retroactivity as being the result of more rapid spread of nerve impulses from very intense stimulations since the discrepancy of the intervals is too great.

VOL.
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196C

In 1939 and 1952 [9, 12], I noted the phenomenon of retroactivity using less intense stimuli and explained it on the grounds that sensation as a reflex act is a process gradually formed in time and in the formative stage before reaching the level of complete stability various factors may still influence it. The possibility is opened up of utilizing this method of investigating the dynamics of maturation of sensations in micro-intervals of time for quantitative biophysical measurements on the development of sensation and its time characteristics. Sensation and its passages in the central nervous system—the cerebral cortex—arising in response to brief stimulation, in our case, in response to light stimulation with a duration of 0·9 msec, last much longer than the stimulation producing it.

In 1949, Moruzzi and Magoun [15] and Bremer [16] formulated the concept of an "ascending reticular activizing system". We now distinguish specific and unspecific afferent systems.

The unspecific afferent system includes the reticular formation of the pons of the diencephalon, the tegmentum of the basal section of the mesencephalon, the subthalamus and the intralaminar and reticular nuclei of the optic thalamus. This unspecific afferent system is obviously involved to a greater degree the stronger the stimulation exerting a diffuse influence on the cortex as observed in the experiments indicated.

Brief very intense stimulation has its own special features; it does not damage the excitable substrates because of its brevity but its effect cannot be reduced solely to that of bringing into activity a large number of excitable elements.

Investigation of the dynamics of human sensations on stimulation of the sense organs in micro-intervals of time opens up the possibility of quantitative biophysical investigation of maturation of sensations and their interaction.

## SUMMARY

(1) Very intense light stimulation, 200,000 lx on the pupil of the eye (10″ adequate optical rheobases) with a duration of 0·9 msec caused a shift in the light excitability and electrical excitability of the eye, exceeding in duration by hundreds of times the duration of the very intense optical stimulation.

(2) The shift in the functional state in response to very intense light stimulation was observed not only in the visual but in other analysers—auditory and skin but its intensity and duration were less than in the directly stimulated visual analyser.

(3) This shift in the functional state has all the features of an adjusting reflex, manifest in a measurable shift in the functional state both of the stimulated analyser itself and the other analysers not directly stimulated; obviously because of the participation of the reticular system.

(4) Motokawa investigated only part of the phenomenon—the second phase of the after-effect of optical stimulation occurring in a macro-interval of time (seconds) whereas in micro-intervals of time (milliseconds) changes are also observed in the form of reduced excitability, occurring immediately after very intense stimulation and lasting 300–500 msec.

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* There is a translation into English of this work.

# RELATION BETWEEN ABSOLUTE LIGHT THRESHOLD AND THE RATE OF RISE OF A STIMULUS LASTING LESS THAN THE "USEFUL" TIME*

T. M. KROL'

Ukhtomskii Physiological Institute, Leningrad

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VOL. 5 1960

THE present work is devoted to study of the excitability of the human optic analyser and elucidation of its dependence on one of the parameters of the stimulus—the "gradient factor".

Previous laboratory work [1, 2] has shown that the excitability of the visual analyser is differentiated and is maximal at a definite combination of intensity, duration and spatial distribution of stimulus.

From the theory of differentiated excitability and adequacy evolved by it may also be assumed that the rate of rise of the light stimulus is not immaterial to the value of the light threshold. However, experimentally this problem has received very scant attention. Lasareff [3] and Drew [4] investigated the relation between different light thresholds and the rate of rise of stimulus; Fröhlich [5] noted the effect of the stimulus gradient on the duration of the latent period and the length of the sensation and Ronchi and Bittini [6, 7] have pointed to the dependence of the critical frequency of fusion of flickers on the shape of the stimulus.

In recent years there have appeared many papers dealing with the relation between the electrical reaction of the retina (e.r.g.) and the form and rate of rise in the intensity of stimulus [8–11]. However, there are very few papers in the literature devoted to study of the effect of the stimulus gradient on the value of the absolute light threshold [8, 12].

In a previous communication [13] we investigated the importance of the gradient of light stimulation for stimuli the duration of which was longer than the "useful" time and showed that the DuBois-Reymond gradient law applies to the effect of an optical stimulus; the stimulating effect of light was greater the steeper the increase in the stimulus in time.

The purpose of the present work, a continuation of the preceding one, was to ascertain whether this relationship applies to the effect of stimuli, the duration of which is less than the "useful" time.

* Biofizika 5: No. 6, 685–690, 1960.

## METHODS

The main experiments on the dependence of light threshold on the stimulus gradient were carried out with the arrangement shown in Fig. 1. This apparatus makes it possible to vary the light stimulus in intensity, duration and gradient and to direct the stimulus to a definite selected region of the retina. We used a Nagel adaptometer (Fig. 1) to ensure delivery to the observer's eye of light stimulation graduated in intensity. In the front wall of the adaptometer an opaque metallic plate (5) was inserted, having only one vertical slit (6) measuring 1 × 20 mm. The light rays passing through this slit were collected by lens (7), passed through the diffuse filters (10) and impinged on the inspection disk (8) located at the centre of the arc of the perimeter.

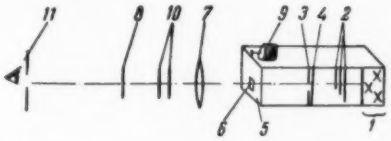


FIG. 1. Experimental layout. 1, illuminator; 2, darkener; 3, Aubert diaphragm, 4, ground glass; 5, metallic plate; 6, slit; 7, lens; 8, inspection disk; 9, electric motor; 10, light filters; 11, artificial pupil.

A moveable fixation point was placed on the perimeter ensuring stimulation of the selected region of the retina.

An electric motor (9) was fixed on the upper surface of the adaptometer. A cardboard circle was mounted on its shaft, arranged parallel to the slit (6) and between it and lens (7). In the cardboard circle there was a cut-out (or several cut-outs) depending on the nature of the experiment. The cut-outs were so made that on rotation of the shaft of the electric motor they coincided with slit (6) and the light fell on the observer's eye only when the cut-out passed in front of the slit (6). We had a set of carboard circles with cut-outs of different length and by changing the speed of rotation of the electric motor, we were able to vary the duration of the light stimulus over a wide

FIG. 2. From of light stimuli. Explanation in text.

range. By making non-uniform cuts in the cardboard rings (Fig. 2), the rate of rise and intensity of stimulation could be altered. When cut-out "a" passed by slit (6) the subject's eye received a light stimulus increasing instantaneously and maintaining a constant intensity during the entire period of its action. When the cut-out "b" passed by the slit the subject's eye was acted upon by a stimulus, the intensity of which gradually and uniformly increased.

Since the object of the present work was to clarify the significance of the gradient factor for stimuli, the duration of which was less than the "useful" time, in our work a comparison was made only of the thresholds for stimuli of equal duration but differing in gradient. We assessed the significance of the gradient factor from the value $\alpha_{(t)}$, i.e. the ratio of the threshold of a gradually increasing stimulus $R_{(t)}$ to the threshold of an instantaneously increasing stimulus $R_0$ of the same duration

$$\alpha_{(t)} = \left(\frac{R}{R_0}\right)_t$$

All the experiments were carried out after 40–50 min preliminary dark adapation. The dimension of the object in the majority of experiments was 3° and only in individual control observations varied from 7° to 11. The main experiments were carried out with an artificial pupil of 2 mm. All the observations were made monocularly. The fovea centralis was fixed by means of a red fixation point which, in most of the experiments, was at a distance of 7–10° from the test object and in the control observations was from 2° to the boundary of the visual field. Three well-trained subjects took part in the work — females aged from 19 to 26 years.

VOL. 5 196

## RESULTS

In the first series of experiments the thresholds were determined by rhythmic stimuli differing solely in gradient. The instantaneously and gradually increasing stimulations were applied to the inspection disk alternately (as Fig. 2 shows). The duration of the stimulus varied from 0·01 to 3 sec. The results of the observations were recorded kymographically (Fig. 3). The kymogram showed the number of rhythmic stimulations received by the eye per unit time and the number of responses of the subject for the same time i.e. Marakov's technique was used [14]. The lower marker in our kymograms recorded the delivery of gradually rising stimuli, the middle one the instantaneously increasing stimuli and the upper one the responses of the subject.

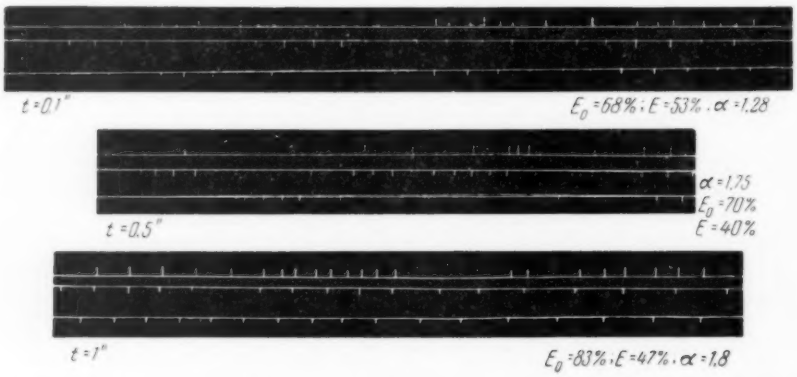


FIG. 3. Kymographic recording of results of experiments in which rhythmic light thresholds were determined by stimuli differing only in gradient. Explanation in text.

The intensity of the stimulus was that at which the subject noted about 70–80 per cent of the total number of instantaneously increasing stimuli. Fig. 3 shows the results of a typical experiment performed on one of our subjects — O. V. On the left of each kymogram is indicated the duration of the stimulus, on the right, the excitability for the stimuli with instantaneous rise ($E_0$), and for the gradually increasing ones ($E$).

The excitability in relation to the rhythmic stimuli was determined from the formula [14]

$$E_t = \frac{b}{Q(i, T, q, t)}$$

where $b$ is the number of light sensations seen by the subject in time $t_0$ and $Q$ is the number of light stimulations received by the subject in the same time at a given intensity of light ($i$), duration of flash ($T$) and given area of illumination ($q$).

Analysis of the findings for our subjects shows that excitability to rhytmic light stimulations of the same frequency, duration and energy depends on the stimulus gradient. Excitability to an instantaneously increasing stimulus is greater than to one of gradual rise; the difference in excitability which we determined from the relation $E_0/E = \alpha$ is greater the longer the stimulus lasts.

In the second series of experiments the light thresholds were determined with single stimuli of equal duration differing in gradient. During the experiments 20 threshold determinations were made; 10 for the gradually increasing alternately and 10 for the instantaneously increasing stimuli.

Experiments with each duration of stimulus were repeated several times and the results were treated statistically for each subject separately (an example, for subject O. V. — 19 years of age — is given below). For the instanteneously and gradually increasing stimuli of the same duration we determined the arthmetical means of the thresholds ($M_1$ and $M_2$), the standard deviations ($\sigma_0$ and $\sigma_2$) and the standard errors ($\overline{m}_1$, and $\overline{m}_2$). Then, by Student's method, $P$ — the probability of the null hypothesis — was determined and from the absolute value $\alpha = M_2/M_1$ and $P$ we estimated the presence or absence of a difference in the value of the thresholds for instaneously and gradually increasing stimuli of the same duration.

The results of statistical treatment of the data showed that with an increase in the duration of the stimuli $\alpha$ rises and the probability of the null hypothesis diminishes.

TABLE

| $T$ sec | $M_1$ | $\sigma_1$ | $\overline{m}_1$ | $M_2$ | $\sigma_2$ | $\overline{m}_2$ | $t$ | $P$ | $\alpha = M_2/M_1$ |
|---|---|---|---|---|---|---|---|---|---|
| 0·03 | 4563 | 410 | 128 | 4731 | 440 | 138 | 1·26 | $0·2<P<0·3$ | 1·04 |
| 0·05 | 3311 | 321 | 100 | 3493 | 354 | 112 | 1·2 | $0·2<P<0·3$ | 1·05 |
| 0·1 | 1939 | 176 | 55 | 2053 | 188 | 59 | 1·4 | $0·1<P<0·2$ | 1·06 |
| 0·5 | 1486 | 150 | 47 | 1630 | 278 | 68 | 1·75 | $0·05<P<0·1$ | 1·10 |
| 1·0 | 1109 | 139 | 44 | 1282 | 203 | 64 | 2·23 | $0·02<P<0·05$ | 1·16 |
| 3·0 | 951 | 130 | 41 | 1146 | 178 | 57 | 2·8 | $0·001<P<0·01$ | 1·20 |

For stimuli, the duration of which varied for our subjects from 0·5–0·05 sec (on average 0·1 sec) the probability of the null hypothesis was so great that there was no point in speaking of a reliable difference in thresholds for instantaneously and gradually nicreasing stimuli.

Our findings for these stimuli only allow us to speak of the presence of a tendency to an increase in difference between thresholds of stimuli of equal duration differing in gradient. This tendency is increased with an increase in the duration of the stimulus. The experimental findings obtained in this series of experiments may be approximated by the curves from the equation:

$$\alpha = 1 + a(t - b)^{p/q}$$

where $\alpha$, the ratio of the threshold of gradually increasing stimulus to the threshold of instantaneously rising one, $t$, the duration of the stimuli, $a$, $b$ and $p/q$ are constants with $1 > \frac{p}{q} > 0$.

For the case given in the Table, $a = 0{\cdot}128$, $p/q = 0{\cdot}35$ and $b = 0{\cdot}1$.

## DISCUSSION

At the start of the present work we sought to determine on the basis of the theory of differentiated excitability and adequacy developed by Makarov [1, 2], whether the effectiveness of the light stimulus depends on the speed of its increase.

In a previous work [13], it was shown that an adequate light stimulus, the duration of which is longer than the "useful" time is more effective the more rapidly it rises. Does this relation also hold true of the effect of short light stimuli the duration of which is less than the "useful" time? The experiments outlined above performed both with single and with rhytmic stimuli show that the significance of the gradient factor for the stimuli of different duration was not uniform within the limits of 0·01 sec to 3 sec. For stimuli of duration less than 0·1–0·2 sec (varying for different subjects) the difference between the thresholds of the instantaneously and gradually increasing stimuli was low and statistically insignificant.

For stimuli with a longer duration, the abruptly using stimulus has an advantage over the gradually increasing one and this advantage is greater the longer the duration of the pair of stimuli under comparison.

In explaining our findings we set out from the hypothesis of Hecht [15], the electrophysiological investigations of Adrian [16], Hartline [17] and others and also from the theory elaborated by Makarov [1].

According to the hypothesis of Hecht the photoreceptors of the retina vary in sensitivity and an increase in illumination leads to an increase in the number of acting receptors. Hence, it is clear that if a light stimulus rises gradually then during the time when its intensity is still increasing, elements are excited differing in sensitivity. The receptors with the greatest light sensitivity react the first and begin to adapt themselves under the influence of constinuing stimulation.

At this time the stimulus continuing to intensify reaches the level of the threshold of other light sensitive elements which, in turn, start to adapt themselves, and so forth. Thus, the central nervous system receives a flow of impulses attenuated and extended in time, which, in the view of Makarov and Gol'burt [18] is not very effective in producing a threshold effect for stimulation of the central sections of the visual analyser. If the retina is acted upon by an instantaneously increasing stimulus, an immediate response is given to it by all the light sensitive elements for which this stimulus is threshold or above threshold. The impulses flow into the central nervous system in a continuous dense formation. Such a stimulus is more effective for stimulation of the central sections of the visual analyser [18].

It is clear from these remarks that the difference in the value of the thresholds for instantaneously and gradually increasing stimuli must be greater the longer the duration of the pair of stimuli under comparison. However, if the duration of the stimulus is close to or less than the time of the "on-effect" (0·1 sec) then all the light sensitive elements excited by this stimulus respond at once, irrespective of the form of the stimulus and the impulses travel to the central nervous system simultaneously. The effectiveness of the stimulus will depend only on its energy. Thus, on the basis of electrophysiological findings, Hecht's hypothesis and the theory of differentiated excitability of Markov, we can explain the independence of threshold from the stimulus gradient found in our experiments for light stimulations of duration close to 0·1 sec or shorter and also, the advantage of the rectangular shaped stimulus over the gradually increasing one for durations less than the useful time but graeter than 0·1 sec.

This explanation of the experimental data is only a working hypothesis and does not rule out other possible interpretations of our findings.

## SUMMARY

On comparing the absolute light thresholds to stimuli differing solely in gradient and lasting less than the useful time but greater than 0·1 sec (on average) it was found that an instantaneously increasing stimulus is more effective than a gradually increasing stimulus of the same duration. For stimuli shorter than 0·1 sec (on average) it is unimportant whether the light stimulus increases instantaneously or gradually. This is true both for single and for rythmic stimuli.

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* There is a translation into English of this work.

VOL. 5 1960

# SIGNIFICANCE OF THE FACTORS OF INTENSITY, DURATION AND AREA IN DETERMINING THE EXCITABILITY OF THE HUMAN VISUAL ANALYSER*

P. O. MAKAROV and T. M. KROL'

Zhdanov State University, Leningrad

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PROBLEM of relationship between the intensity and duration and also between the energy and duration of threshold light stimuli has long attracted the attention of biophysicists, physiologists and ophthalmologists. While on the first problem all workers unanimously confirm the longstanding conclusion of Exner [1] on the presence of a hyperboloid relation between intensity and duration and have only supplemented and defined it, there is as yet no unanimity of opinion on the problem of the relation between the energy of threshold light stimuli and their duration. In contrast to the findings of a number of workers [2–5] who have shown that with a reduction in duration, the energy of the light threshold stimuli passes through a minimum, Blondell and Ray [6], Long [7], Luizov and Kuznetsova [8] failed to detect any minimum but found that the energy rises linearly with increase in the duration of stimulation.

It is interesting to note that the findings of the workers who have detected a minimum for the energy are not uniform. Thus, Pigron [3] found a minimum at 2 msec, Fedorova and Grushetskaya [4] at 5 msec and Vishnevskii and Tsyrlin [9] at 50 msec. The discrepancies in the findings of these workers led Luizov [8, 10] to doubt altogether the presence of a minimum of energy.

A point which attracts our attention is that the workers who have been occupied with elucidating the relation between energy and duration of adequate light stimuli worked with objects of different angular size. Can this perhaps explain the discrepan-

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cies in their findings? The present work, in confirmity with the findings of Graham and Margaria [11], is aimed at answering this question.

## METHODS

The experiments were carried out using a Makarov adequatometer. The instrument consists of an illuminator, an adaptometer, the tube of which passes from the subject's room to the experimenter's room and a pendulum with a contracting and expanding slot. The subject sits at the inspection disk at one end of the adaptometer tube, the experimenter at the other. The pendulum swinging between the illuminator and adaptometer, imparts to the adaptometer a known amount of light corresponding to the width of the slot. Knowing the velocity of swing of the pendulum and the width of the slot, the time for which the light acts on the subject's retina can be determined. The intensity of the light is graduated by means of light filters and an iris diaphragm and the area of the test object by means of a diaphragm mounted on the sight glass. The control for this diaphragm and its scale were in the room with the experimenters. Fixation was ensured by a red fixation point, in the frontal plane. All the experiments were carried out monocularly after dark adaptation for 40–60 min. The nasal half of the retina was stimulated at a distance of 5–10° from the fovea centralis along the horizontal meridian. Control experiments were carried out with an artificial pupil of 2 mm. Two well-trained female subjects took part in the work—E. Sh. and P. N., 21 and 27 years old respectively. The measurement procedure was as follows. For a test object of a definite area we found the threshold of intensity (adequate optical rheobase—AOR) then by increasing successively the AOR, 1, 2, 3, 4, 10, 20 and 40 times the thresholds of duration corresponding to these intensities were determined. The measurements were carried out for the following angular dimensions of the test object; 5°, 1°, 30′ and 11′. From the data obtained for the different angular dimensions of the test object, strength—duration curves and curves relating the energy of the threshold stimuli to their duration were plotted. The energy was computed from the formula $E = It$, where $I$, intensity of the stimulus in rheobases, $t$, the threshold of duration in msec corresponding to the given intensity.

## RESULTS

Tables 1 and 2 give the data obtained after statistical treatment of the experiments for each individual subject. Each line presents information on only one intensity; in the graphs the results obtained for a definite area of the test object (*Rh* in AOR) are given.

The experimental results were treated as follows; for each value of intensity of stimulus (with unchanged area) we found the mean threshold of duration in msec ($M$) and the standard error ($m$) determined from the formula

$$m = \sigma / \sqrt{n}, \text{ where } \sigma = \pm \sqrt{\frac{\Sigma(v - M)^2}{n}}$$

where is number of measurements and $v$ is the variant.

TABLE 1

| I | 5° | | | 1° | | | 30′ | | | 11′ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M± (msec) | t | P | M± (msec) | t | P | M± (msec) | t | P | M± (msec) | t | P |
| 1·3 *Rh* | 900±139·0 | 6.7 | <0·001 | 2344±339·0 | 7·0 | <0·001 | 3800±250·0 | 15·2 | <0·001 | 6800±603·0 | 9·6 | <0·001 |
| 2·0 „ | 90±5·0 | 18·0 | „ | 160±19·0 | 8·5 | „ | 240±22·0 | 10·9 | „ | 320±46·0 | 7·0 | „ |
| 4·0 „ | 35±2·0 | 17·5 | „ | 59±4·0 | 15·0 | „ | 83±6·3 | 13·2 | „ | 110±10·0 | 11·0 | „ |
| 10·0 „ | 18±1·2 | 15·0 | „ | 26±3·0 | 8·7 | „ | 34±3·3 | 10·3 | „ | 50±7·0 | 7·1 | „ |
| 20·0 „ | 9±0·6 | 15·0 | „ | 14±1·4 | 10·0 | „ | 16±1·4 | 11·4 | „ | 27±3·5 | 8·0 | „ |
| 40·0 „ | 6±0·3 | 20·0 | „ | 8±0·5 | 16·0 | „ | 10±1·0 | 10·0 | „ | 12±1·2 | 10·0 | „ |

*Subject E. Sh.

TABLE 2

| I | 5° | | | 1° | | | 30′ | | | 11′ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M± (msec) | t | P | M± (msec) | t | P | M± (msec) | t | P | M(± msec) | t | P |
| 1·3 *Rh* | 1800±414 | 4·3 | <0·01 | 3400±380 | 9·0 | <0·001 | 5000±377 | 13·2 | <0·001 | 5800±438 | 13·2 | <0·001 |
| 2·0 „ | 105±9·5 | 11·0 | <0·001 | 208±16 | 13·0 | „ | 260±27 | 9·6 | „ | 380±25 | 15·2 | „ |
| 4·0 „ | 42±4·3 | 9·8 | „ | 30±6 | 13·3 | „ | 112±15 | 7·5 | „ | 117±9 | 13·0 | „ |
| 10·0 „ | 25±2·4 | 10·4 | „ | 41±4 | 10·2 | „ | 50±5·5 | 9·0 | „ | 70±8 | 8·7 | „ |
| 20·0 „ | 13±10 | 13·0 | „ | 20±1·6 | 12·5 | „ | 20±3·0 | 8·6 | „ | 40±4·1 | 9·7 | „ |
| 40·0 „ | 8±0·6 | 13·3 | „ | 10±1·1 | 9·1 | „ | 13±1·1 | 11·8 | „ | 20±1·9 | 10·5 | „ |

*Subject P.N.

VOL. 5 1960

Next, we determined the reliability of the mean. According to Student the criterion for establishing the reliability of the mean is not the absolute value of its error but the relation between the mean and its error, $t = M/m$. From the value $t$ and the number of degrees of freedom $n'$ ($n' = n - 1$) we determined from Student's table the probability with which the sample mean may be outside the limits of the population mean. Consideration of the data presented in Tables 1 and 2 shows that $P$ is less than 0·001 for nearly all cases. This suggests that the mean values obtained in our experiments are quite reliable.

We shall try, on the basis of the data in Tables 1 and 2, to evaluate the relation between the strength–duration curve and the area of the test object.

We note first of all that in our experiments reduction in the area of the stimulus inevitably led to an increase in the threshold of duration (see any of the lines in Tables 1 and 2) and this, despite the fact that the threshold intensity of the stimuli was constant. The phenomenon described above occurred for all the intensities investigated (1·3, 2, 4, 10, 20 and 40 AOR).

Next, if we increased the intensity of the stimulus whilst keeping its area constant (see any of the columns in Tables 1 and 2) then the threshold of duration fell off and the curve of the relation between intensity and duration of stimuli was of the usual hyperboloid form [1, 12–15].

Do the strength–duration curves determined for the stimuli of different angular dimension differ among themselves? We sought to evaluate this difference from the reciprocal of the difference between the threshold of duration for an intensity of 1·3 AOR and the threshold of duration at 40 AOR. This ratio, a characteristic of the slope of the curve, we denote by $k$; $k = \dfrac{1}{x_2 - x_1}$ where $x_1$ is the threshold of duration for 40 AOR and $x_2$ for 1·3 AOR. The function $k$ for each area of stimulus used in our experiments are given below (multiplied, for convenience, by $10^5$)

| Observer | 5° | 1° | 30′ | 11′ |
|---|---|---|---|---|
| E. Sh. | 111·8 | 42·8 | 26·4 | 17·2 |
| P. N. | 55·8 | 29·5 | 20·0 | 17·3 |

These calculations show quite clearly that the slope of the strength–duration curve does not remain constant with change in the area of stimulus.

We now turn to consideration of the relation between the energy of the threshold stimuli, on the one hand, and their intensity and duration on the other (Figs. 1 and 2).

We note first of all that with a reduction in duration, the energy of the threshold stimuli ($It$) passes through a minimum. With a reduction in the angular dimension of the test object the minimum becomes broader (except for the curve for 11′ obtained for subject P. N.), with a tendency for the energy of the threshold stimuli to fall on further reduction in their duration.

With regard to the relation between the energy of the threshold stimuli and the intensity (Figs. 1(b) and 2(b)) in subject P. N. the minimum for all areas has 4 rheobases and in subject E. Sh. the smaller the area the greater the intensity of the stimulus (rheobases) to which the minimum corresponds.

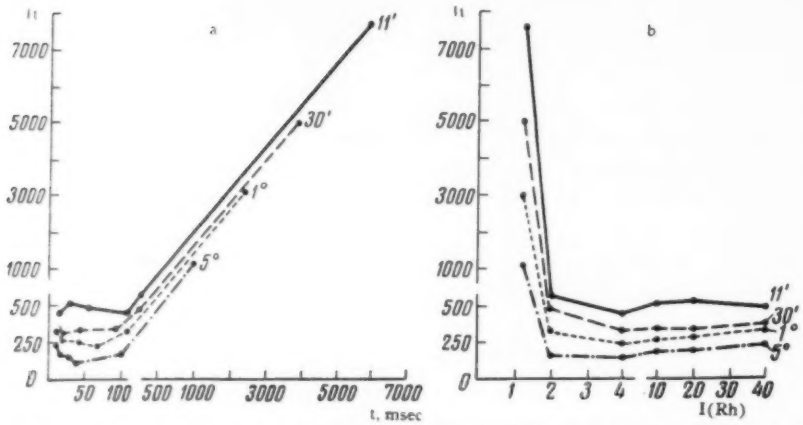


FIG. 1. Relation between energy of threshold stimuli of different angular dimensions and their duration (a) and intensity (b). Findings for subject E. Sh. (aged 21) with normal vision. Abscissa, duration on stimuli in msec (a) and their intensity in rheobases (b); ordinates, energy of threshold stimuli (*It*). Gaps along axes denote changes in scale.

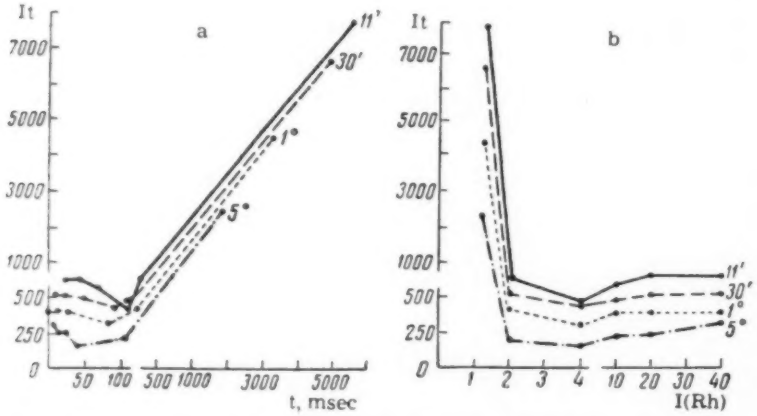


FIG. 2. Relation between energy of threshold stimuli of different angular dimensions and their duration (a) and intensity (b). Findings for subject P. N. (aged 27) with normal vision.

## CONCLUSION

Thus, the results of the experiment reported above in which a relation was found between the course of the energy curve and the area of the stimulus suggests that the difference in the findings in the literature is to be explained by the fact that many investigators worked with test objects of different areas. In addition, some workers average the data for a number of observers in whom the minimum relates to different durations and thereby blur the picture of the dependence of the energy of threshold stimuli on their duration.

## SUMMARY

(1) In two subjects an investigation was made of the relation between the intensity of duration and also between the energy and duration of the threshold light stimuli of different angular size.

(2) It was found that the relation between the intensity and duration of the light stimulus did not remain constant with change in its area; the smaller the angular dimension of the test object, the steeper the change in the threshold of time with change in the intensity of stimulus.

(3) With a reduction in the duration of the stimulus, the energy of the threshold stimuli ($It$) passes through a minimum. However, the smaller the angular dimension of the test object, the more indistinct the minimum and the more distinct the tendency for the energy of the threshold stimuli to fall with a reduction in their duration.

(4) It is argued from the results obtained that the conflicting nature of the data in the literature on the problem of the relation between the energy of threshold stimuli and their duration derives from the fact that the authors worked with test objects of different angular dimensions.


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# SYSTEM OF COLOUR VISION OF THE FLY *CALLIPHORA**

G. A. MAZOKHIN-PORSHNYAKOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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THE present work is a continuation of the series of investigations of the colour vision of insects [1–3] and is aimed at a colorimetric study of the colour vision of the faceted eye of *Calliphora erythrocephala* based on electrophysiological appraisal of the visual equality for the insect of test radiations. Several attempts to study the reactions of the eye of *Calliphora* to colours [4–9] have already been published, but their overall results were insufficient to gain an understanding of the system of colour vision of this fly. The more detailed account, given below of the results of the preceding workers will show the advantages accruing from the colorimetric method used by us and the novel aspects of the information on the mechanism of the colour vision of *Calliphora* obtained by this method.

The calorimetric and electrophysiological apparatus used in this work has been already described in detail in connexion with similar experiments on the dragon-fly *Libellula* [2] and the house fly [3]. The main experiments consisted of a careful search for a pair of radiations (the primary) indistinguishable to the fly from a spectral colour and the measurement of the energy of each component of the pair. These colour equalities were found by varying the intensity of the primary radiation so that it was indistinguishable to the fly from the spectral radiation, i.e. its eye did not react, in the electroretinogram, in response to rapid substitutions of these radiations one by the other. If the spectral radiation could not be matched in the way described with one primary alone, then the latter was mixed with a second primary radiation. Two primary radiations proved sufficient to equalize with one or if necessary, with a mixture of any monochromatic radiations.

Such colour equalities were matched for 24 points of the spectrum with $\lambda$ from 365 to 710 m$\mu$ relative to violet ($\lambda$ = 420–455 m$\mu$) and red ($\lambda$ = 630–680 m$\mu$) primary radiations. In other words, all 24 monochromatic colours were expressed for the fly eye by certain ratios of the intensities of the same primary colours of the colorimeter. This made it possible to compute the so-called colour combination curves; functions $a_1(\lambda)$ relative to violet primary colour and $a_2(\lambda)$ relative to red. The functions $a_1(\lambda)$ and $a_2(\lambda)$ were computed from the relation:

$$a_1(\lambda) = \frac{E_1}{E_\lambda} \text{ and } a_2(\lambda) = \frac{E_2}{E_\lambda}$$

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where $E_1$ and $E_2$ are the relative energies of the violet and red primary radiations and $E_\lambda$ is the relative energy of the monochromatic radiation being measured.

Fig. 1 depicts two combination curves representing the mean values of $a_1(\lambda)$ and $a_2(\lambda)$ for about 100 colour equalities obtained on 10 specimens of male and female *Calliphora*. The scatter of the values $a(\lambda)$ as a rule was not in excess of 4–15 per cent of the measured value.

The work of the eye of *Calliphora* at high and low luminosities displays special features similar to the day and twilight vision of man, which indicates the duality of the apparatus of vision of the faceted eye. At low intensities (10–20 lx) the fly does not distinguish all visible spectral radiations from one primary radiation if its intensity is suitably selected. Thus, by changing the intensity of the violet primary radiation it was possible to match it for the fly eye with any spectral radiation from $\lambda$ 365 to 650 m$\mu$; the longer wave red rays of low inten sity no longer excite the eye of the fly. The experiments yielded a two-humped curve characterizing the spectral sensitivity of this "twilight" apparatus of vision (curve (a) Fig. 1).

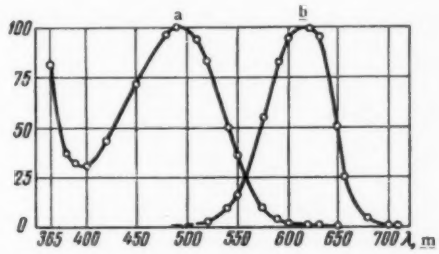


FIG. 1. Curves of spectral sensitivity of the two receptors of faceted eye of the fly *Calliphora erythrocephala*; (a) curve of spectral sensitivity of shortwave receptor of twilight vision; (b) same for longwave receptor of daylight vision. Ordinates: relative sensitivity (for equi-energetic spectrum) as percentage of maximum value of $a(\lambda)$ for each curve.

For a higher intensity of radiations (over 20 lx) the spectrum visible to the fly extends to the long wave side beyond 710 m$\mu$. With one (violet) primary only the short wave (365–500 m$\mu$) monochromatic radiations can be matched. The colorimeter did not give sufficient intensity of monochromatic radiations with $\lambda < 520$ m$\mu$ to excite both receptors. Therefore, for $\lambda$ 500 and 490 m$\mu$ only one-dimensional equalities were found although the second receptor is probably sensitive to these wavelengths. The longwave spectral radiations in the interval $\lambda$ 520–650 m$\mu$ are indistinguishable for the fly only from a definite mixture of two primary radiations, violet and red. The far red rays ($\lambda > 650$ m$\mu$) can be matched for the fly again with one, in this case the red, primary. Consequently, for relatively high intensities the spectral sensitivity of the eye can be colorimetrically characterized by only two colour combination curves (curves (a), (b) Fig. 1), computed from two-dimensional colour equalities. Curve (a) obtained from two-dimensional colour equalities fully coincided with that computed from the one-dimensional equalities in the preceding case when the experiments were conducted at low

light intensities. The two-dimensional colour equalities, the result of using not more than two different primary radiations, demonstrate the existence in the eye of only two types of light receptors (hereafter called receptors) i.e. the presence in the fly of a dichromatic system of colour vision. The daytime apparatus of colour vision, consisting of two receptors, functions only at high luminosities. With twilight illumination only one of its components works—the receptor more sensitive to light and representing the monochromatic apparatus of twilight vision. Such duality of the apparatus of vision well explains certain aspects of the behaviour of *Calliphora* in the optomotor drum described in detail by Schneider [7].

With reference to the colour vision system of the eye of *Calliphora*, it is easy to demonstrate that the colour combination curves obtained with proper choice of the spectral composition of the primary radiations are at the same time the curves of spectral sensitivity of the receptors. Thus, the spectrum visible to the fly has at its opposite ends quite wide one-dimensional regions ($\lambda$ 365–490 and 650–710 m$\mu$) within which all the monochromatic radiations can be matched with one primary by changing only its energy. It is natural to consider that in each of such one-dimensional regions only one receptor functions. In the experiments, the primary radiations, for which the combination curves were obtained, were chosen precisely in the one-dimensional regions of the spectrum, i.e. each primary radiation excited only one type of receptor. Hence, it is understandable that in all the visual colour equalities the energies of the primary radiations were inversely proportional to the sensitivity of the receptors to the given spectral rays. Therefore, the colour combination curves for the fly eye for a given choice of primary radiations are simultaneously the curves of spectral sensitivity of the receptors. For convenience these curves (see Fig. 1) have been plotted as percentages to give an equal scale. The purpose of the colorimetric method is not to compare the sensitivity of the individual receptors to light of the same wavelength and therefore an arbitrary scale may be adopted.

Not only the spectral sensitivity but also other properties of the receptors of the eye of *Calliphora* differ. The short wave or twilight receptor ((a) in Fig. 1) is capable of substantial adaptation and works over a very wide range of light intensities. It takes in the region of the spectrum from ultra-violet to near-red inclusive, although its sensitivity to red rays is negligible; for $\lambda$ 600 m$\mu$ it equals 1·1 per cent and for $\lambda$ 650 m$\mu$ 0·1 per cent (of the maximum in the blue-green). The curve of sensitivity of the twilight receptor has two peaks; one at 490 m$\mu$, the other in the ultra-violet at $\lambda \sim$365 m$\mu$ [9]. (For the equi-energetic spectrum the maximum of the curve corresponds to the point of the spectrum with $\lambda$ 490 m$\mu$ and the second peak reaches 83 per cent of this. If this curve is recalculated for the equi-quantum spectrum the point of the curve for $\lambda$ 365 m$\mu$ exceeds the "maximum" in the blue-green part of the spectrum by about 20 per cent.) The position of the peak in ultra-violet was not accurately established by us because of the difficuties obtaining of monochromatic rays with $\lambda < 365$ m$\mu$. The sensitivity of the receptor at the point of inflexion of the curve ($\lambda$ 400 m$\mu$) was 30 per cent. At low luminosity this receptor is an apparatus for twilight vision and at higher luminosities works simultaneously with the second receptor forming together with it the di-

VOL. 5 1960

chromatic system of daylight vision. The curve of spectral sensitivity of the twilight receptor is quite close to the curve of absorption of certain variants of visual purple [3].

The longwave or daylight receptor functions only at relatively high luminosities. It is apparently poorly, or even not at all adapted to the dark. The daylight receptor covers the region of the spectrum from approximately $\lambda$ 490 to 720 m$\mu$ and has a maximum corresponding to the point of the spectrum with $\lambda$ 620 m$\mu$. For rays with $\lambda$ 572 or 650 m$\mu$ the sensitivity is 50 per cent of this maximum.

The system of colour vision of *Calliphora* exactly coincides with that of the house fly [3]. Therefore, all that has been said by us on the discrimination of colours by the house fly fully applies to the eye of *Calliphora*. For the dichromatic system of vision of these insects there exist no colours not present in the solar spectrum. All mixed colours representing for man white, grey or purple are indistinguishable for the fly from certain monochromatic colours of the portion of the spectrum in which the two receptors operate simultaneously. However, white or purple radiation will be for the fly visually identical with a certain monochromatic radiation only for a strictly definite ratio of intensities of both. Thus, to disturb the fly eye preparation with the highest contrast sensitivity it sufficed to change by 3–4 per cent the energy of one of the components of the colour equality.

What new facts are provided by this colorimetric investigation—as compared with preceding investigations of the colour vision of *Calliphora* in which different methods were used? Most workers have concentrated their attention on the visibility (brightness) curve, in other words, the curve of the relative spectral sensitivity of the eye, seeking to deduce from it the number of receptors and the region of the spectrum in which they function. This curve was found in electrophysiological experiments by the threshold method [4] or from the equality of e.r.g. to different monochromatic radiations [6, 8–10]. All workers are agreed that the visibility curve, if the experiment is performed with high intensity radiations, has three peaks; in the ultra-violet between $\lambda$ 341–369 m$\mu$, in the green ~507 m$\mu$ and in the red ~630 m$\mu$ (Fig. 2) and in the equi-quantum spectrum the peak in the ultra-violet is higher than the other two. The visibility curve determined for a lower level of brightness has only the two first maxima and the rise in the red is either absent or barely noticeable.

Resorting to additional experiments or advancing certain indirect arguments individual workers offered different explanations for the origin of the separate peaks of the visibility curve. Autrum and Stumpf [5, 6] applied the method of "heterochrome flickers" i.e. substituting various "equi-bright" (from the visibility curve) spectral radiations. They established that in the e.r.g. ($\lambda_1/\lambda_2$-potential) the eye reacts to the flickers of most pairs of radiations holding this to be evidence of the existence of colour vision in *Calliphora*. From an analysis of the height of the $\lambda_1/\lambda_2$ potential for different pairs of radiations these workers concluded that in the spectral range from $\lambda$ 400–690 m$\mu$, the fly eye has two receptors with sensitivity maxima at about 520 and 630 m$\mu$. This is not inconsistent with the fact that the $\lambda_1/\lambda_2$ potential is absent on replacing the "white" light of an incandescent lamp by monochromatic yellow with $\lambda$ 580 m$\mu$.

Subsequently, from the fact that the peak of the visibility curve at about $\lambda$ 630 m$\mu$

is detected only on strong illumination and that the ommatidia of the eye of *Calliphora* are separated by red pigment, Autrum [10] ascribed the origin of this peak to the transparency of the separating pigment to red light. In the view of Autrum [10], in the green and orange–red part of the spectrum the same receptor works with a sensitivity maximum at about 511 m$\mu$. The rise in the visibility curve in the red is linked with the anatomical

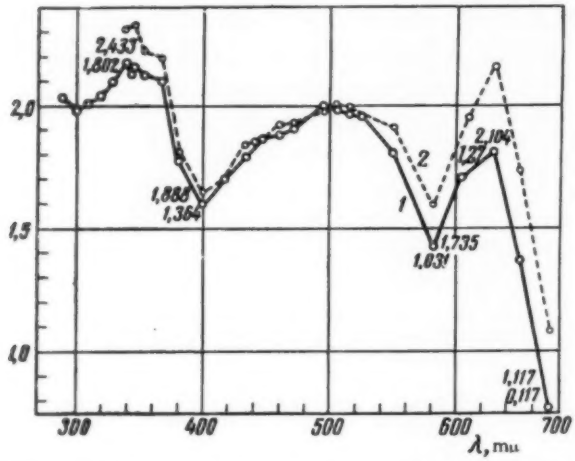


FIG. 2. Relative spectral sensitivity (1/Q) of complex eye of *Calliphora erythrocephala*. Unbroken line, mean values of 24 measurements (numerals denote limits of scatter); broken line, mean values of earlier measurements [8]. Ordinates, log reciprocal of the number of quanta of monochromatic rays giving e.r.g. "on" effect of equal height; abscissa, $\lambda$ in m$\mu$ (after Walther and Dodt [9]).

structure of the eye and not with the presence of an independent receptor for the red end of the spectrum.

Walther and Dodt [8, 9] making a special study of the origin of the third peak (in the ultra-violet) of the visibility curve of *Calliphora* concluded that in the eye of this fly there exist two separate receptors; one in ultra-violet with a maximum between 341–369 m$\mu$, the other in another part of the spectrum with a sensitivity maximum at 507 m$\mu$. In relation to the peak in the red they agree with Autrum's explanation [10]. The main proof offered by Walther and Dodt [8, 9] for the existence of two receptors is the two-peak nature of the visibility curve in the interval $\lambda$ 300–600 m$\mu$. Indirect evidence of this is provided by: (1) the fluctuation sometimes observed (in separate measurements and only for some specimens of flies [8, 9]) in the relative height of both peaks with change in the level of intensity of the spectrum; and (2) the doubtful existence of a photopigment, the absorption curve of which has two maxima, the greater of which lies in the ultra-violet region of the spectrum.

It is not hard to see that given formal agreement in the evaluation of the number of receptors, the basic conclusions derived from colorimetric and the other experimental methods diverge sharply. Such divergence stems from the fact that the conclusions in the first case are the direct result of the colorimetric experiments, whereas in the

other it rests on experimental data (visibility curves) which only indirectly characterize the system of colour vision.

If the fly in colorimetirc experiments does not visually distinguish ultra-violet radiation from blue-green, but distinguishes both these radiations from red, this directly suggests that in the red part of the spectrum an independent receptor functions with no separate receptor in the ultra-violet. But the number of peaks in the visibility curve and their position in the spectrum does not necessarily indicate the number and spectral sensitivity of the eye receptors.

The point is that although the colour combination curves (in colorimetry) have a strict physical meaning, the interpretation of the visibility curve for a multi-dimensional system of vision is very indeterminate. The visibility curve is the curve of the generalized, in some way as yet unknown, spectral sensitivity of all receptors and in defining it a very large scatter of the measured values is always observed [8–10]. Again, a serious handicap to both qualitative and quantitative conclusions based on the visibility curve is the violation of the addition law on combining heterochrome intensities. The non-additivity of the human visibility curve is expressed in the fact that two "equi-bright" radiations do not remain so after addition to them of a third radiation. Experiments on insects have not been carried out in this form; however, the non-additivity of the visibility curve of *Calliphora* plainly follows from certain of Autrum's [10] experiments. Thus, the curves for the different spectral radiations relating the height of the "on" effect of e.r.g. (in mV) to the number of light quanta are not parallel but intersect. This also means that for *Calliphora* there is not a single curve which would characterize the uniform visual intensity of all spectral radiations irrespective of their energy.

Therefore, it is not necessary to consider that the number of receptors must be reflected precisely in the number of peaks and not in some other aspect of the shape of the visibility curve. Man, as is known, has three receptors but the photopic visibility curve has one peak.

However, it should be noted that only for a multidimensional system of vision is it impossible to characterize completely the ability to react to light by any one value. When one receptor functions, then any means of determining its response to radiation (from the visibility curve or from the combination curve) characterizes the same property of the receptor—its spectral sensitivity. This is also clearly apparent in the case of the visibility curve for *Calliphora*. Only the left ($\lambda$ 365–550 m$\mu$) and the right ($\lambda$ 630–680 m$\mu$) part of the visibility curve (see Fig. 2) coincide with the portions of the curves of spectral sensitivity of the receptors of the fly eye since only one receptor functions for each of these wavelengths. It is impossible to determine each spectral sensitivity for the same wavelength in the central part of the visibility curve which corresponds to the region of the spectrum where the two receptors function simultaneously.

In the example of the fly, already knowing the number of receptors and their spectral sensitivity, it was possible in part to interpret the visibility curve of the eye. The opposite problem—finding the number of receptors and the regions of the spectrum occupied by them from the visibility curve—is not always feasible.

We now have to consider the two other arguments of Walther and Dodt [9] to the

effect that the peaks of the visibility curve of *Calliphora* in the ultra-violet and blue-green reflect the presence of two separate receptors.

The ambiguity of the link between the height of the maxima of the visibility curve in the ultra-violet and blue-green noted in some experiments [8, 9] may be explained from the properties of the twilight receptor. This receptor rapidly and thoroughly adapts itself. Therefore, it is very difficult to make a set of measurements of the sensitivity while maintaining unchanged the state of adaptation of the eye and it is not easy to distinguish a minor adaptatory shift from the case in which the shape of the visibility curve actually changes when measured in various conditions of brightness. A similar objection to colorimetric experiments is untenable, since in such experiments the so-called colorimetric conditions of matching are automatically respected [11].

Finally, the difficulty of equating the two-peak curve of spectral sensitivity of the twilight receptor of *Calliphora* with the light absorption curve of any known photo-pigment, in our view, is not a sufficient reason for holding that such a receptor cannot exist.

Some workers [12, 13] completely explain the high sensitivity of insects to ultra-violet light by the fluorescence of the eye (chitin lens and light insulating pigments)—under the action of these rays. However, such a view of the mechanism of the sensitivity of the eye to ultra-violet is inconsistent with the laws of physics. On fluorescence one quantum of ultra-violet cannot give rise to more than one quantum of light in the visible region of the spectrum. Therefore, the sensitivity of the eye (twilight receptor) to ultra-violet radiation with $\lambda$ 365 m$\mu$ could not be higher than to any other spectral radiation with longer $\lambda$. In fact, the maximum in the ultra-violet for equi-quantum spectrum exceeds by about 20 per cent that for 490 m$\mu$. Moreover, if the sensitivity of the eye to ultra-violet were merely due to fluorescence of the ocular media, one-dimensional colour equalities in the interval $\lambda$ 365–500 m$\mu$ would be impossible for the eye of *Calliphora* since the fluorescence of insect eyes is mixed—it contains together with blue and green, yellow and orange [14].

## SUMMARY

The colorimetric method [2, 3] has been used to determine the spectral sensitivity curves of the two receptors of the faceted eye of *Calliphora* forming a dichromatic system of colour vision (Fig. 1). The shortwave receptor functions over a spectral range from the ultra-violet to rays with $\lambda$ 650 m$\mu$, and has two maxima of sensitivity; near 365 and 490 m$\mu$. The sensitivity of the second longwave receptor encompasses $\lambda$ 490–720 m$\mu$, its maximum lies at 620 m$\mu$. For low luminosity (to 10–20 lx) only one, more light-sensitive and rapidly adapting shortwave receptor functions—the twilight apparatus of vision of the fly. At high luminosities both receptors function simultaneously.

The advantages of colorimetry over other methods of study of the colour vision of insects are examined.

VOL. 5 1960

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# DECOMPRESSION PHENOMENA IN THE HUMAN BODY IN CONDITIONS OF EXTREMELY LOW ATMOSPHERIC PRESSURE*

P. N. Ivanov, A. G. Kuznetsov, V. B. Malkin and Ye. O. Popova

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Interest in the study of decompression phenomena occurring in the body at extremely low barometric pressure arises from the requirements involved in man's attempts to master flight in the upper strata of the atmosphere.

The studies [1–3] established that in animals in a medium with low barometric pressure (below 47 mm Hg), rapid development of vapour and gas formation is observed in different parts of the body; under the skin, in the connective tissue separating groups of muscles, in the cavity of the heart and pericardium and in the abdominal and pleural cavities. Some have termed this phenomenon "decompression tissue emphysema" [4], others "cosmic boiling of fluids".

Chemical analysis of the content of the bubbles of subcuteneous emphysema made by a number of workers [2, 3] has shown that they are almost completely filled with water vapour. This confirmed the previous assumption that the onset of altitude subcutaneous emphysema is due to the boiling of tissue fluids. There were grounds for believing that these phenomena could be accounted for solely by the laws of physics. However, in a number of studies the importance of physiological factors has been demonstrated. Thus, Kuznetsov [4] noted that the resistance of different species of warm-blooded

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animals to reduced barometric pressure varied. For example, in rabbits the onset of altitude subcutaneous emphysema could be observed in almost every experiment, but in dogs in the same conditions it was noted much less often.

Besides species differences, the importance of individual variations in the resistance of animals (rats) to the development of altitude emphysema has been established [2]. This author advanced the hypothesis that the difference is due to the differing degree of turgor of the tissues in different animals.

Of great theoretical and practical interest is the study of this phenomenon in human experiments.

A number of workers in investigations with humans at barometric pressures of 47 mm Hg and lower have noted the development of altitude tissue emphysema in the region of the wrists not protected by altitude appearel or other types of altitude equipment. Agadzhanyan, Vakar, Mansurova and Tsivilashvili [6] studied the development of altitude subcutaneous emphysema in the wrists; it was shown that it could be prevented by wearing special gloves producing in the wrists a back pressure equal to 50 mm Hg.

VOL. 5 196

But, in other studies known to us the observations on the development of altitude emphysema were, as a rule, incidental to altitude research. Special attention has not been paid to the development of decompression phenomena.

The purpose of the present work was to study the course of development of decompression phenomena at extremely low baromeric pressures and to assess their effect on the general state and work capacity of man. An attempt was made to find out the significance of certain factors in the onset of altitude tissue emphysema.

The work was done with the participation of six normal males aged from 20 to 30 years. In the pressure chamber rarefaction for a short interval of time was produced from 760 to 41–8·5 mm Hg. A special assembly of oxygen equipment gave a high level of oxygen supply to the subjects, and, over the whole surface of their bodies, except for the wrists sufficiently high pressure was produced.

To determine the character and localization of the decompression phenomena expressed in the formation of gas or vapour in the body tissues, the wrists were X-rayed in the ulnar and volar projections before the "ascent", at "altitude" (in the first minute, then every 3–5 min) and on "descent". At the sites of the most frequent appearance of altitude emphysema of the wrists the skin temperature was taken (two points on the dorsal surface of the wrist and one point of the palmar surface in the region of the main thumb joint of the right and left hands). The temperature was measured by a resistance microthermometer which was first calibrated against a mercury thermometer, graduated in 0·1°.

In 25 investigations the subjects every 5–10 min performed static work—for one minute they held a load of 15 kg with both hands.

To assess the physiological shifts and the general state of the subjects a six-channel oscillograph was used to record the electroencephalogram, electrocardiogram, respiratory movements of the thorax and, by means of an oxygenometer, the saturation of arterial blood with oxygen were determined.

In 38 of 72 investigations made at reduced barometric pressure (41–8·5 mm Hg)

the appearance of altitude subcutaneous emphysema was observed. Its onset was indicated by sudden thickening of the wrist, and in the radiographs by the appearance in the region of the soft tissues of translucent layers of different size and intensity.

The onset of emphysema did not impair the sense of well-being and general state of the subjects which enabled us to observe the course of its development for some time. However, as a rule, a certain time after the onset of emphysema the subjects complained of pains in the region of the wrist of the affected hand. The pain sensations usually appeared 3–5 min after the visible onset of subcutaneous emphysema. In rare cases complaints of unpleasant sensations in the wrist coincided with the visible appearance of emphysema. However, in no case in which the presence of signs of emphysema were detected only by radiography did the subjects complain of pain in the hand. In individual cases, the painful sensations did not occur even with highly marked emphysema.

As the observations and results of radiographic examination showed, subcutaneous emphysema developed a certain interval of time after the drop in barometric pressure. The radiographs showed that the development of emphysema usually started on the dorsal surface of the wrist. It was localized either only in this region or rapidly spread along the dorsal and lateral surfaces of the wrist, sometimes even embracing the phalanges of the fingers, except for the terminal ones. Sometimes it appeared also in the region of the distal part of the forearm. With considerable development of emphysema translucent patches could even be detected on the radiographs between the metacarpal bones. The distance between the dorsal surface of the metacarpal bones and the surface of the skin in the median part of the wrist reached 12–19 mm when the development of emphysema was pronounced.

The resistance of the subjects to altitude emphysema was found to differ. Thus, on rarefaction to 41 mm Hg subcutaneous emphysema was observed only in 1 of the 6 subjects. When the barometric pressure dropped to 8·5 mm Hg the development of emphysema was noted in as many as 5 subjects, appearing rarely in 2 of them and in nearly all the investigations in one.

A striking aspect was that with a decrease in the barometric pressure there was a rise in the number of cases of subcutaneous emphysema. Thus, of 18 investigations made at a rarefaction of 41 mm Hg emphysema was observed in 5, whereas of 15 investigations made in conditions of rarefaction of 8·5 mm Hg emphysema was found in 11. Altitude subcutaneous emphysema developed earlier, the lower the barometric pressure. Thus, on lowering the pressure to 41 mm Hg the onset of emphysema in the various investigations started from the 17th minute. A drop in barometric pressure to 8·5 mm Hg caused the appearance of emphysema as from the fourth minute.

Performace of graduated, relatively light static work made no appreciable difference to the development of altitude subcutaneous emphysema. Of definite interest were the instances in which the subjects under uniform experimental conditions (duration of observation and degree of rarefaction) developed emphysema in one case and not in another. Thus, for example, in two investigations emphysema appearing on rarefaction of 41 mm Hg did not develop in an experiment made after several days even when the rarefaction reached 25 mm Hg.

It may be inferred from these observations that the development of altitude emphysema is determined not only by the physical laws but by certain physiological factors varying from one investigation to the next. It was assumed that the inconstancy in the development of this phenomenon is possibly determined by differences in the temperature of the skin of the wrists of the subjects.

Measurements showed that the temperature of the skin of the hands of the subjects during the experiment rose to a varying extent. For example, in some persons it rose by 3–4°, whereas in others in the majority of investigations only by 2° or less. The rise in temperature was due, it must be supposed, to congestive phenomena in the region of the wrists produced by the special apparatus exerting pressure on the fore and upper arm. Fig. 1 shows the temperature curves of the skin of the hands recorded in one of the investigations.

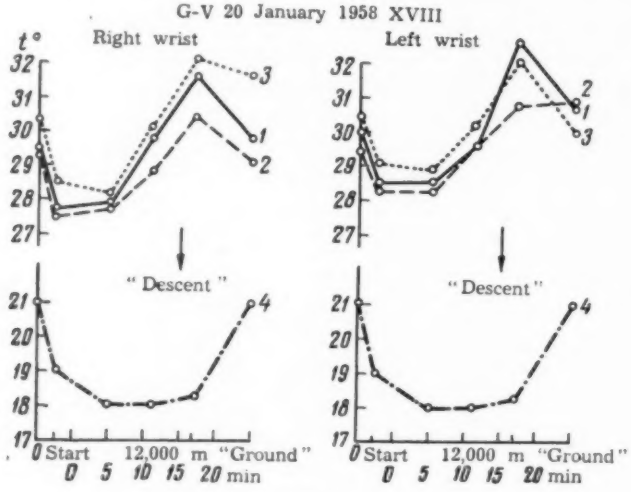


FIG. 1.

Comparison of the temperature curves does not reveal a clear relationship between the onset of altitude emphysema and change in the temperature of the skin of the hands. All that could be noted was that in subject Ch. who showed the greatest rise in temperature of the skin of the hands, subcutaneous emphysema developed more often than in the other subjects. In individual subjects with a uniform rise in the temperature of the skin of the left and right wrists emphysema developed only in one hand. In 3 cases emphysema developed in the subjects' wrist when the temperature of the skin was even somewhat lower than in the wrist not affected by emphysema.

Hence, the results show that the difference in the skin temperature was not the main cause of the individual variations in the appearance of altitude emphysema.

Besides the phenomena of altitude subcutaneous emphysema, the development of which was noted in about half the investigations, in all cases with a fall in barometric pressure to 41 mm Hg or lower gas formation in the radio-carpal joint was noted.

In the radiographs the presence of gas was seen in the form of translucent zones both in the region of the interosseous joint spaces and over the dorsal surface of the bones of the joint. Gas bubbles could be distinctly observed in the region of the ulnar margin of the cuneiform bone.

It is interesting that the signs of gas in the region of the radio–carpal joint were already noted on the first radiograph, taken 15–30 sec after the ascent to "height". Further stay of the subjects at "altitude" insignificantly changed the size of the space occupied by the gas. The appearance of gas in the region of the radio-carpal joint was independent of the development of altitude subcutaneous emphysema.

In radiographs it was shown that in the region of the radio-carpal joint, gas bubbles appeared practically at the same time in the right and left hands, whereas altitude tissue emphysema, as a rule, developed only in one hand and only in rare cases simultaneously in both hands.

Differences between the decompression phenomena in the region of the soft tissues of the wrist and the radio-carpal joint were also detected during gradual lessening of the rarefaction of air.

During the descent from "height", as the barometric pressure increased the dimensions of the altitude emphysema rapidly fell but the translucense in the region of the radio-carpal joint remained comparatively unchanged even at a pressure of 145 mm Hg. After bringing the pressure in the barometric chamber to normal the signs of presence of gas in the radio-carpal joint immediately vanished. In the same conditions in individuals with marked subcutaneous emphysema slight lucent bands could still be seen in the radiograph. These phenomena have been described and explained in a paper by one of the present authors [4].

The radiographs of the wrist of the right hand taken in one of the investigations 30 sec after reducing the barometric pressure to 21 mm Hg showed small translucent foci, suggestive of gas bubbles, in the region of the radio-carpal joint; after 10 min considerable translucense was distinctly visible in the region of the tissues of the wrist, i.e. decompression tissue emphysema was present.

Our findings point to a difference in the course of development of decompression phenomena in the region of the radio-carpal joint and the soft tissues of the wrist. It may accordingly be assumed that the mechanism of development of decompression phenomena in the region of the radio-carpal joint and the soft tissues of the wrist differ.

The rapid appearance at altitude of gas bubbles in the region of the radio-carpal joint is probably due to solubility changes of the gases dissolved in the tissues and not to a process of boiling. Of course, it cannot be ruled out that water vapour then penetrates the gas bubble. However, the volume of gas cannot appreciably increase since the tissues sourrounding the gas bubble have considerable toughness and low elasticity. This obviously explains the relatively constant dimensions of the gas bubbles in radiographs taken at different periods of the subjects stay in conditions of low barometric pressure (41 mm Hg and lower). The regular appearance of gas bubbles in the region of the radio-carpal joint is apparently related to the conditions promoting the formation of embryonic bubbles in the joints, as indicated by many workers [7, 8].

Another interesting point is that despite the fact that in all 72 investigations gas bubbles were noted in the radio-carpal joint, only in 3 cases did the subjects complain of altitude pains in the region of the wrist.

As is known [9], in a number of cases gas bubbles are observed in the subjects' tissues in the absence of any complaints. Burkhard, Adler, *et al.* [9] believe that altitude pains appear only when the gas buble is sufficiently large (5·8 $cm^3$). This contention is not quite warranted since altitude pains are known to occur even on formation in the tissues of extremely small gas bubbles. It seems to us important to call attention again to the fact that the pathogenesis of altitude pains still remains obscure.

In studying the various factors influencing the development of altitude emphysema, serious attention must be paid to the temperature of the tissues and the interstitial pressure. These factors must undoubtedly exert an appreciable effect on the boiling of tissue fluids.

Our experiments show that the changes in temperature in the range investigated do not determine the individual peculiarities in the development of altitude emphysema. Probably, of great importance is the interstitial pressure, the magnitude of which may vary greatly. It is known that this magnitude is not constant and depends on many factors; age, nutrition, specific features of metabolism, functional state of the central nervous system and, in particular, the sections regulating water and salt metabolism. As such, the interstitial pressure is undoubtedly a factor preventing the development of altitude emphysema. For the tissue fluids at body temperature (37°) to start to boil it is necessary that the sum of the values of the interstitial (Pm) and the barometric pressure (Pb) be less than 47 mm Hg ($Pm + Pb < 47$ mm Hg).

The absence of altitude emphysema in man in the first 2–3 min period of reduced barometric pressure (41–8·5 mm Hg) makes it reasonable to suppose that the value of the interstitial pressure of the soft tissues of the wrist (taking into account the above equation) must exceed 35–40 mm Hg; such a pressure is a factor preventing the abrupt boiling of the tissue fluids on lowering the barometric pressure to 41–8·5 mm Hg. It may be assumed from the findings that the development of altitude emphysema as already indicated by Kuznetsov [4] and Balakhovskii [2], probably has two phases. In the first phase the gases ($N_2$, $CO_2$, $O_2$) dissolved in the tissues comes out of solution with the formation of small gas bubbles. Then, when as a result of growth of the bubble a low-pressure zone is formed in the tissues, conditions are created for the boiling of the tissue fluids (start of second phase). In this phase a rapid increase in gas formation is observed with appearance of apparent altitude emphysema.

The following conclusions may be drown from this investigation.

(1) At barometric pressures of 41 mm Hg and lower, the radiographs of all the subjects showed the presence of gas in the region of the radio-carpal joint. Only in 3 of 72 cases were there altitude pains.

(2) Altitude subcutaneous emphysema in conditions of a rarefied atmosphere was noted irregularly; the probability of its onset was greater the lower the barometric pressure. The individual resistance of nominally healthy persons to altitude emphysema was found to vary.

(3) On development of altitude emphysema the general conditions of the subject remained good for several minutes. The appearance of pain sensations was noted, as a rule, not earlier than 3–5 min after onset of emphysema.

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# SPECTROPHOTOMETRIC AND RADIOISOTOPE INVESTIGATION OF PREPARATIONS OF DEOXYRIBONUCLEIC ACID ISOLATED FROM THE RAT LIVER AFTER EXPOSURE TO STRONTIUM 90*

M. S. USPENSKAYA

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IT has been demonstrated that solutions of the sodium salts of deoxyribonucleic (DNA) and ribonucleic (RNA) acids and also of purine and pyrimidine bases after X-irradiation [1], exposure to $\gamma$ and $\beta$ rays from a rhodium source [2], or thus to the $\gamma$ rays of $^{60}Co$ [3] absorb less ultra-violet light thus indicating chamical changes in their molecules. A similar phenomenon has been observed *in vivo* in experiments; spectrophotometric investigation of DNA solutions from the thyroid gland of irradiated rats [4] and also, by us, on liver DNA solutions from rates exposed to polonium [5].

The $^{90}Sr$ entering the body is rapidly and almost completely taken up by bone tissue [6]. Nevertheless, bearing in mind the crucial rôle of the nucleoproteins in the vital activity of the cells, the question has been raised as to whether there occurs a certain binding of $^{90}Sr$ or the radioactive products of its decay ($^{90}Y$) by the nucleic substances of the liver, the components of deoxyribonucleoprotein (DNP)–DNA and the protein

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of DNP. Particular interest has been roused by the possible effect of radioactive isotopes on the structure of the DNA molecule.

An investigation was made of the ultra-violet absorption spectrum in solutions of DNA isolated from rat liver after exposure to $^{90}Sr$. In addition, during developments of radiation sickness, caused by this radioactive substance, measurements were made of the radioactivity of the liver tissue, the DNA preparations and the protein component of DNA isolated from it.

In the experiments we used 25 white male rats weighing 200–250 g which, on an empty stomach, were injected subcutaneously with a solution of $^{90}SrCl_2$ (present in equilibrium with $^{90}Y$) in an amount of 2 mc/kg animal weight. In addition, 5 rats served as control.

The rats were killed by decapitation (4 rats at each stage of the investigation) 1·5 hr, 1, 3, 9 and 13 days after injection of $^{90}Sr$. The liver dissected from the carcase was rapidly washed with saline at 0°, dried with filter paper, weighed and cut into small pieces over ice with scissors. A weighed sample was spread in an even layer on a sample holder for measurement of the radioactivity by the usual method with an end-window counter.

In order to obtain preparations of high polymer DNA in the form of its sodium salt from the liver, the liver was broken up in a homogenizer cooled with ice. The polymer DNA was isolated by Shapot's modification [7] of Mirsky and Pollister's method. The protein component of the DNP was obtained by emulsifying the DNP, isolated by the same method, with a mixture of chloroform and *iso*-amyl alcohol (9:1) with subsequent centrifugation. The protein thus separated from the DNA was repeatedly washed with increasing concentrations of alcohol, then with a mixture of alcohol and either (3 : 1) and then with ether.

After measuring the radioactivity of the liver preparations on the day the animals were killed they were kept on the sample holders and were remeasured at definite intervals of time, starting from the period of the half life (T) of $^{90}Y$—64·8 hr [8]. The radioactivity was expressed in units of $10^{-7}$ millicuries per gramme moist tissue and as the percentage of the injected dose.

The radioactivity of the DNA preparations and the protein component of DNP were measured not on the day of death but after the few days needed for their isolation by the usual method. We were thus able to measure the radioactivity of the DNA preparations after four $^{90}Y$ half lives (4 T) and the radioactivity of the protein preparation after 10 T. An exception was measurement of the DNA preparations and the DNP protein isolated from the liver 1 hr after injecting the rats with $^{90}Sr$. For the purpose of comparison the radioactivity of these preparations was measured at the same times during the 40 days after their isolation i.e. at time intervals of 5, 7, 10, 12 and 15 T (T for $^{90}Y$).

From the DNA preparations isolated from the liver of the control animals and those exposed to $^{90}Sr$, we prepared 0·005 per cent aqueous solutions (pH 6·5) and determined the ultra-violet absorption spectra in the region from 230–300 m$\mu$. The

measurements were made on a SF-4 type spectrophotometer. For measurement of the optical density quartz cuvettes were used in which the thickness of the absorbing layer of test solution was 10 mm.

## RESULTS

Table 1 presents the numerical values of the radioactivity of the liver tissue at various times during development of acute radiation sickness caused by injection of $^{90}Sr$ in equilibrium with $^{90}Y$. These results show that after injection of rats with these radioactive agents, radioactivity appeared in the liver.

TABLE 1. RADIOACTIVITY OF LIVER TISSUE AT DIFFERENT TIMES DURING DEVELOPMENT OF RADIATION SICKNESS DUE TO INJECTION OF $^{90}Sr$ (IN EQUILIBRIUM WITH $^{90}Y$)

| Time after injection of $^{90}Sr$ (+$^{90}Y$) | Number of animals | Activity on day of experiment per g moist tissue | | Activity of preparations after storage for | | | |
|---|---|---|---|---|---|---|---|
| | | $10^{-7}$ mc | % of injected dose | 5 T (13·5 days) $10^{-7}$ mc | 10 T (27 days) $10^{-7}$ mc | 20 T (81 days) $10^{-7}$ mc | 30 T (81 days) $10^{-7}$ mc |
| 1 hr | 4 | 22,161±1551 | 0·444±0·034 | 8930±523 | 8208±605 | 8183±624 | — |
| 5 hr | 4 | 25,911±1488 | 0·537±0·034 | 3605±71 | 2626±72 | 2421+37 | 2375±45 |
| 1 day | 4 | 18,039±1514 | 0·351±0·047 | 1719±100 | 838±57 | 775±55 | 748±52 |
| 3 days | 4 | 4295±511 | 0·082±0·01 | 333±63 | 287±70 | 250±78 | 248±68 |
| 9 days | 4 | 663±41 | 0·014±0·0 | 199±5 | 194±10 | 183±10 | 184±8 |
| 13 days | 4 | 240±18 | 0·005±0·0 | 141±14 | 129±13 | 129±8 | 120±8 |

The maximum radioactivity in the liver was established 5 hr after exposure to $^{90}Sr$ (after 1 hr the radioactivity on average was 0·444 per cent of the injected dose and after 5 hr 0·537 per cent).

The increase in the radioactivity of the liver 5 hr after inecjtion of $^{90}Sr$, as compared with that noted after 1 hr, indicates that at this time the percentage of $^{90}Sr$ to $^{90}Y$ in the hepatic tissue appreciably changed because of the greater accumulation in it of $^{90}Y$. This is borne out by the figures in Table 1 showing the sharper fall in radioactivity of the liver preparations of animals killed 5 hr after exposure when kept for 5, 10, 20 and 30 T (T for $^{90}Y$) in relation to the radioactivity of the liver preparations of rats killed 1 hr after exposure to the radioactive agents.

In the ensuing times (after 1, 3, 9 and 13 days) the radioactivity of the liver sharply fell and by the 13th day only 0·005 per cent of the radioactive substance remained in the liver. The mean radioactivity of the liver tissue 5 hr after injection of $^{90}Sr$ rose at compared with the values established 1 hr after injection by 12·4 per cent and in the subsequent period (1, 3, 9 and 13 days) on the contrary, fell by 18·7, 21·7, 97·0 and 99 per cent respectively.

If we compare the values of the radioactivity in the liver tissue given in Table 1 after 1 and 5 hr and 1, 3, 9 and 13 days with that of the same samples after 5 T we see that in the times investigated it fell in relation to the original radiaoctivity (measured

on the day of the experiment) in each case by 2·5, 6·9, 10·6, 14·3, 3·5 and 1·7 times respectively.

The means results of measurement of the radioactivity of these liver preparations after storage for up to 81 days (Table 1) show that a considerable part of the radioactivity is associated with $^{90}Y$ since only after about a month's storage (to be more accurate, after 10 T for $^{90}Y$) when a large part of the $^{90}Y$ had already decayed did these preparations have a relatively unchanged radioactivity due to the presence of $^{90}Sr$ ($+^{90}Y$) retained in the hepatic tissue.

The results of the determination of the radioactivity of preparations of polymer DNA and the protein component of DNP from the liver of the rat exposed to $^{90}Sr$ ($+^{90}Y$) 1 hr after injection and for different storage times are given in Table 2.

It will be seen that the preparations of DNA and DNP protein isolated from the rat liver 1 hr after exposure to $^{90}Sr$ possessed radioactivity. Comparison of the radioactivity of the preparations at the indicated storage times revealed that the radioactivity of DNA after a time equal to 10 T (T for $^{90}Y$) did not change whereas the radioactivity of the DNP protein measured at periods of the half life of $^{90}Y$ continued to fall in each case.

VOL. 5 1960

TABLE 2. RADIOACTIVITY OF DNA PREPARATIONS AND PROTEIN COMPONENT OF DNP OF LIVER OF RATS KILLED AN HOUR AFTER INJECTION OF $^{90}Sr$ ($+^{90}Y$) IN A DOSE OF 2 mc/kg

| Time of measurement of activity of preparation after killing animals | | Activity per mg dried preparation $10^{-7}$ mc | |
|---|---|---|---|
| Days | T for $^{90}Y$ | DNA | DNP protein |
| 13·5 | 5 | 1·437±0·307 | 1·907±0·239 |
| 18·9 | 7 | 0·428±0·065 | 0·483±0·140 |
| 27·0 | 10 | 0·200±0·095 | 0·233±0·040 |
| 32·4 | 12 | 0·209±0·085 | 0·114±0·009 |
| 40·5 | 15 | 0·216±0·070 | 0·098±0·009 |

Thus, the results of these determinations show that $^{90}Sr$ ($+^{90}Y$) injected into rats could within only 1 hr be detected in the DNP of the liver and its daughter product $^{90}Y$ apparently at this time was more firmly fixed by the DNA than by the protein. The activity of the latter during storage of the preparation fell more appreciably than that of DNA.

Table 3 shows the radioactivity of the DNA preparations isolated from the rat liver at different times during the development of radiation sickness after exposure to $^{90}Sr$. It was established that the greatest radioactivity was found in the DNA preparations isolated from the liver 5 hr after injection of $^{90}Sr$, i.e. in the period of the greatest accumulation of yttrium in the organ. On the first and third days their radioactivity was considerably less. On the other the findings in Table 3 hr show that the radioactivity of the DNA preparations isolated 5 hr and 1 and 3 days after injection of strontium fell, on storage from 4 T to 7 T (T for $^{90}Y$), from interval of one T to another in most cases by about a half, which points to the presence in DNA of yttrium-90.

At the same time, radioactivity of the DNA preparations isolated 1 hr after exposure of the rats to strontium fell appreciably more slowly on storage; this is apparently related to the presence in DNA at this time of $^{90}Sr$ ($+^{90}Y$).

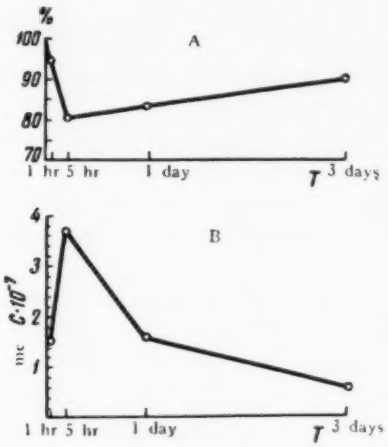


FIG. 1. A, average results of change in optical density of solutions of DNA preparations isolated from liver of rats exposed to $^{90}Sr$. Abscissa, time of isolation of DNA after injection of $^{90}Sr$ in hours and days; ordinates, optical density of DNA solutions as percentage of normal (taken as 100 per cent). B, radioactivity of DNA preparations isolated from rat liver at various times after injection of $^{90}Sr$ (in dose of 2 mc/kg body weight). Ordinates, radioactivity per mg dried DNA preparation from liver units of $10^{-7}$ mc.

Of great biological interest was the investigation of the absorption spectra of the DNA solution of rat liver exposed to $^{90}Sr$ since these investigations allow one to from an idea of the degree of chemical disturbances in the polymer molecule.

The upper curve in Fig. 1 depicts the mean data (on the basis of investigations of preparations obtained from 4–5 animals at each period of the investigation) characterizing the changes in the optical density of the solutions of the DNA preparations of the rat liver exposed to $^{90}Sr$. These results are expressed in percentages of the

TABLE 3. RADIOACTIVITY OF POLYMER DNA PREPARATIONS ISOLATED FROM RAT LIVER AT VARIOUS TIME AFTER INJECTION OF $^{90}Sr$

| Time of isolation of DNA after injection of $^{90}Sr$ ($+^{90}Y$) | Number of animals | Activity of DNA in $10^{-7}$ mc per mg dried preparation | | | |
|---|---|---|---|---|---|
| | | Measured on isolation after | | | |
| | | 4 T (10·8 days) | 5 T (13·5 days) | 6 T (16·2 days) | 7 T (18·8 days) |
| 1 hr | 8 | 1·560±0·303 | 1·437±0·229 | 0·550±0·087 | 0·428±0·065 |
| 5 hr | 3 | 3·850±0·019 | 1·495±0·311 | 0·855±0·010 | 0·395±0·064 |
| 1 day | 3 | 1·590±0·147 | 1·016±0·097 | 0·503±0·095 | 0·210±0·035 |
| 3 days | 3 | 0·576±0·085 | 0·120±0·060 | — | — |

normal taken as 100. They show that the greatest fall in optical density at the absorption maximum (260 mμ) in relationto the norm was given by the solutions of DNA preparations isolated 5 hr after injection of $^{90}Sr$.

The lower curve in Fig. 1 shows the radioactivity of the DNA preparations for the same times of investigation.

Comparison of these curves shows that the size of the fall in the optical density of the DNA solutions is related to the level of radioactivity of the DNA preparations isolated from the liver at given times after injection of $^{90}Sr$. Thus, the DNA preparations isolated 5 hr after injection of $^{90}Sr$ had maximum radioactivity and the solutions showed the greatest fall in optical density in the ultra-violet region of the spectrum. Conversely, the DNA preparations isolated from the liver 1 hr and 3 days after injection of the radioactive substance displayed less radioactivity and the optical density of their solutions was correspondingly higher.

Another point to note is that the direction of the curve of radioactivity of the DNA preparations isolated from the liver at different times after injection of $^{90}Sr$ is in full accord with the curve of change of the radioactivity of the liver tissue as a whole (Fig. 2). It may be assumed that the profound changes in optical density of the DNA solutions were due not only to the inherent radioactivity of DNA but to the radioactivity of the cells of the liver tissue as a whole.

VOL. 5 1960

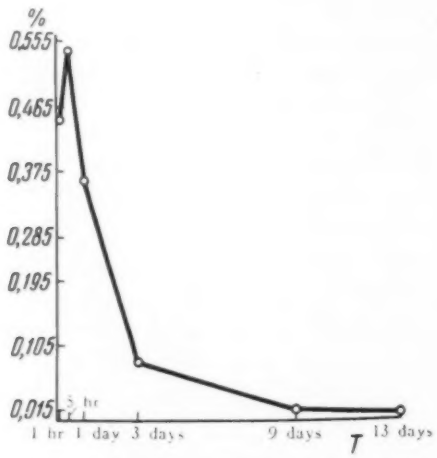


FIG. 2. Radioactivity of liver tissue of rats exposed to $^{90}Sr$ in a dose of 2 mc/kg body weight (subcutaneously). Abscissa, time of investigation after injection of $^{90}Sr$ in hours and days; ordinates, radioactivity per g moist liver tissue as percentage of injected dose.

The results of determinations of the radioactivity of the protein component of DNP isolated from the rat liver at different times after injection of $^{90}Sr$ are given in Table 4. It was established that the preparations of the protein component of DNP, as with the DNA preparations, possessed radioactivity. While the maximum incorporation of the radioactive isotopes in the DNA preparations was detected 5 hr after their

entry into the body, the maximum incorporation of these substances into the DNP protein was noted after 3 days. On the ninth day the activity of the protein component of DNP fell appreciably and after 13 days was half that on the ninth day.

TABLE 4. RADIOACTIVITY OF THE PROTEIN COMPONENT OF DNP PREPARATIONS ISOLATED FROM RAT LIVER AT VARIOUS TIMES AFTER INJECTION OF $^{90}Sr$

| Time of isolation of protein after injection of $^{90}Sr$ ($+^{90}Y$) | Number of animals | Activity in $10^{-7}$ mc per mg dried protein | | | |
|---|---|---|---|---|---|
| | | 10 T (27 days) | 20 T (54 days) | 25 T (67·5 days) | 30 T (81 days) |
| 1 hr | 3 | 0·233±0·040 | 0·104±0·009 | — | — |
| 5 hr | 4 | — | 0·150±0·032 | 0·138±0·026 | ± |
| 1 day | 4 | 0·266±0·023 | 0·251±0·023 | 0·250±0·017 | 0·229±0·203 |
| 3 days | 4 | 0·316±0·027 | 0·305±0·019 | 0·307±0·038 | 0·290±0·060 |
| 9 days | 4 | 0·174±0·095 | 0·153±0·022 | 0·153±0·043 | 0·142±0·036 |
| 13 days | 4 | 0·077±0·004 | 0·075±0·014 | 0·065±0·002 | 0·061±0·014 |

Measurement of the radioactivity of protein in this series of experiments was made 27, 54, 67, 81 days (i.e. at the end of 10, 20, 25 and 30 T) after isolation. As our results show, the slight decrease in radioactivity on storage of the protein preparations isolated 1, 3, 9 and 13 days after incorporation of $^{90}Sr$ ($+^{90}Y$) was apparently due to the presence of $^{90}Sr$. Consequently, a small amount of this radioactive elements, as stated above, was retained in the liver for as long as 13 days; in this period the radioactivity of the protein component of DNP was still manifest.

## SUMMARY

(1) Solutions of the DNA preparations of the liver obtained from rats exposed to $^{90}Sr$ (in equilibrium with $^{90}Y$) absorb less ultra-violet light (260 m$\mu$) and the greatest decrease in optical density of DNA solution was manifest 5 hr after injection of the radioactive isotopes.

(2) The preparations of high polymer DNA isolated from the rat liver 1 and 5 hr and 1 and 3 days after injection of $^{90}Sr$ ($+^{90}Y$) possessed radioactivity; the maximum radioactivity of DNA was noted 5 hr after injection.

(3) The maximum radioactivity of the protein component of DNP of the liver was detected 3 days after exposure of the animals to $^{90}Sr$.

(4) The greatest accumulation of radioactive isotopes in the liver occurred 5 hr after their injection into the body.

(5) The radioactivity of the DNA preparations, the DNP proteins and the intact liver tissue after exposure to $^{90}Sr$ ($+^{90}Y$) was due to the presence in them both of $^{90}Sr$ and $^{90}Y$; the relation between these radioactive elements in the preparations tested varied with the period of observation.

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* There is a translation into English of this work.

VOL. 5 196

# DISTURBANCE OF OXIDATIVE-REDUCTION PROCESSES IN PLANT TISSUES UNDER THE INFLUENCE OF IONIZING RADIATION*

A. M. KUZIN and V. A. KOPYLOV

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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IN previous papers one of us [1–3] showed that after X-irradiation of plants of *Vicia faba* an aqueous extracts of the irradiatied leaves produced a much lower mitotic index in the meristem tissue of normal rootlets of this plant than did corresponding extracts from unirradiated leaves.

These observations indicate that the effect of irradiation (10–20 Kr) is to produce disturbances in the normal metabolism of foliar tissue. It may be assumed that these disturbances lead to changes in the concentration of normal metabolites, or to the appearance of anomalous substances, causing delay in division of the meristem cells. In the present work we attempted to elucidate the disturbances in metabolism which occur in the leaves of *Vicia faba* after irradiation.

All the experiments were undertaken with 13–15-day old plants of *Vicia faba.* The batches of plants grown in normal conditions were divided into halves; one served as control and the other was irradiated by an RUP unit. Conditions of irradiation: dose rate 400 r/min, time 25 min, dose 10,000 r. During irradiation the plants were

* Biofizika **5:** No. 6, 716–719, 1960.

kept in the same conditions as the controls. The leaves immediately or 24 hr after irradiation (depending on the object of the experiment) were ground up in distilled water with iron-free quartz sand in the cold (+4°C), ratio of leaves to was 1 : 1. The extraction was carried out in distilled water in a ratio of 1 : 5. The aqueous extract of the irradiated leaves rapidly darkened on contact with the atmospheric oxygen, where in the aqueous extract of the control plants this process occurred much more slowly.

From the amount of stained substances formed, we estimated in rate of oxidation. The measurements were made on a FEK-M apparatus without light filter (Table 1).

TABLE 1. RATE OF OXIDATION OF EXTRACTS FROM NORMAL AND IRRADIATED LEAVES. DOSE 10,000 r
(Mean results of five experiments)

| Time, min | Extinction | | Time, min | Extinction | |
|---|---|---|---|---|---|
| | Normal | Irradiated | | Normal | Irradiated |
| Immediately | 0·02 | 0·02 | 60 | 0·08 | 0·12 |
| 30 | 0·02 | 0·02 | 90 | 0·12 | 0·16 |

A notable feature in this respect is that oxidation started after a certain induction period which, as is known, is characteristic of tyrosinase.

The qualitative tests with ferric chloride for phenols in the aqueous extract gave a distinctly greater content of them in the irradiated leaves than in the controls. In order to satisfy ourselves that in the irradiated plant tissues intensification of oxidative processes actually occurs as a result of molecular oxygen we measured, by the manometric method, the absorption of oxygen by homogenates of the control and irradiated leaves in a Warburg apparatus. The results of the experiment are given in Table 2.

TABLE 2. RATE OF OXIDATION OF HOMOGENATES OF IRRADIATED LEAVES, AS PERCENTAGE OF NORMAL, AT VARIOUS INTERVALS AFTER IRRADIATION OF LEAF
(Estimated from uptake of oxygen during 30 min in a Warburg apparatus)

| Time, hr | % of normal | | | Time, hr | % of normal | | |
|---|---|---|---|---|---|---|---|
| | Radiation dose, r | | | | Radiation dose, r | | |
| | 100 | 1000 | 10,000 | | 100 | 1000 | 10,000 |
| 4 | 106 | 109 | 117 | 32 | 104 | 125 | 176 |
| 24 | 108 | 140 | 198 | 48 | 105 | — | 118 |

As the results show, the leaves irradiated with a dose of 1000 r gave an increased absorption of $O_2$ 4 hr after irradiation reaching 140 per cent after 24 hr. For a dose of 10,000 r these changes were even more distinct; 24 hr after irradiation intensification of the oxidative processes was up to 200 per cent. On potentiometric measurement of the pH in the homogenates, a certain shift to the acid side, as compared with the controls, was always observed. Thus, pH measurements of the homogenates of normal plants gave 6·5–6·3 whereas the figures for the homogenates of the irradiated plants

were 5·5–5·8. The shift to the acid side in the homogenates of the irradiated leaves indicates intensification of oxidative processes under the influence of irradiation.

These results allow us to assume that in the irradiated leaves the enzymatic processes of oxidation of the phenols are considerably activated. To elucidate the nature of the oxidative process we studied the rate of oxidation of various phenols under the influence of the aqueous extracts of the irradiated and control leaves. In the experiments we used pyrocatechol, pyrogallol, hydroquinone and tryosine. The method of determination has been described [4–6]. The results are given in Table 3.

TABLE 3. INTENSITY OF OXIDATION OF VARIOUS PHENOLS IN EXTRACTS OF IRRADIATED LEAVES AS PERCENTAGE OF CONTROLS
(Mean data of three experiments)

| Phenol | % of normal (norm 100%) | Phenol | % of normal (norm 100%) |
|---|---|---|---|
| Tyrosine | 220 | Pyrogallol | 107 |
| Pyrocatechol | 139 | Hydroquinone | 92 |

VOL. 5 196

The findings show that under the influence of irradiation the process ofoxidation of tyrosine was most sharply intensified. From these observations it would appear that oxidation of tyrosine occurs in the tissues of irradiated plants, which ought in the first place to lead to formation of diphenols passing then into physiologically active quinones—in line with the oxidation of tyrosine by tyrosinase according to Raper [7]. In order to verify this assumption we carried out a series of experiments on the activity of the enzyme tyrosinase in normal conditions and after X-irradiation.

For the experiment we took plants of *Vicia faba* at different periods of the year; January–February, April–May and June. The activity of the enzymatic process in the spring-summer period as a rule is more intensive than in the winter period. As stated above, the conditions of cultivation for all experiments were identical. Although the activity of tyrosinase changed with the period of the year, the irradiated plants always gave higher tyrosinase activity than the unirradiated ones.

The tyrosinase activity was determined photocolorimetrically from the increase in the stained products [5] at $t = 37°C$. The results of the measurements of one of the series of experiments are shown in Table 4.

TABLE 4. RATE OF OXIDATION OF TYROSINE ACTED ON BY TYROSINASE IN IRRADIATED LEAVES. DOSE, 10,000 r

| Time (min) | Extinction | | | | | |
|---|---|---|---|---|---|---|
| | Normal | | | Irradiated | | |
| | Experiment No. | | | Experiment No. | | |
| | 1 | 2 | 3 | 1 | 2 | 3 |
| Immediately | 0·05 | 0·04 | 0·05 | 0·04 | 0·06 | 0·06 |
| 30 | 0·05 | 0·04 | 0·05 | 0·04 | 0·06 | 0·06 |
| 60 | 0·12 | 0·12 | 0·13 | 0·32 | 0·34 | 0·35 |

For the quantitative determination of the quinone we tested with ferrous oxide in the presence of potasium ferro-cyanide and an excess of oxalic acid. The amount of Prussian blue formed was determined colorimetrically on a FEK-M apparatus. The following results were obtained (Table 5).

TABLE 5

| Experiment No. | Time after irradiation | Extinction | |
|---|---|---|---|
| | | Normal | Experiment |
| 1 | 20 min | 0·03±0·01 | 0·10±0·02 |
| | 24 hr | 0·04±0·02 | 0·16±0·03 |
| 2 | 20 min | 0·04±0·02 | 0·09±0·02 |
| 3 | 20 min | 0·03±0·01 | 0·08±0·02 |
| | 24 hr | 0·03±0·01 | 0·15±0·03 |

The results make it clear that quinone-like substances were formed in the leaves soon after irradiation and increased regularly during the subsequent 24 hr. The increases shown immediately after irradiation in the oxidative processes decreased with time. Thus, for example, the activity of tyrosinase 24 hr after irradiation was 1·8 times higher than the controls, whereas immediately after irradiation it was 3·2 times.

The abnormally high concentration of the quinone-like products of oxidation of tyrosine, formed on irradiation may exert an effect on the intracellular structures and, in particular, may be responsible for the arrest of cell division usually observed on irradiation of plants. The nature of these substances in now being studied by us.

## SUMMARY

(1) The metabolic processes in leaves after X-irradiation with a dose of 10,000 r. have been investigated.

(2) It has been shown that immediately after irradiation the pH of the homogenates of the irradiated leaves shifts to the acid side in relation to the controls.

(3) Raised susceptibility to oxidation by atmospheric oxygen of the homogenates of the irradiated leaves, as compared with the unirradiated ones, has been demonstrated.

(4) Raised activity of tyrosinase in irradiated leaves has been shown.

(5) An increased content of quinone-like substances in the irradiated leaves, as compared with the controls, has been demonstrated.

(6) Increases, though diminishing in time, were distinctly manifest 24 hr after irradiation.

(7) The hypothesis is advanced that the products of quinoid nature formed on oxidation of tyrosine may be responsible for the depression of cell division usually observed on irradiation of plants.

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# LOCAL REACTION OF TISSUE TO IRRADIATION*

A. D. STRZHIZHOVSKII

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VOL. 5 1960

THE mitotic activity and life span of tissue cells may be regulated both by local and central factors. The first category includes the functional state of the interacellular structures and the interrelation of the cells during their vital activity [1]. Central regulation is mediated by special regulatory mechanisms of a nerve-humoral character [2]. Moreover, the intensity of cell division and the life span of the cell are affected by the ability of the body as a whole to provide the cell with the substances it requires, i.e. its general state.

Accordingly, local and distant influences can be distinguished in radiation damage to the tissue. The former represent the result of direct damage by radiation to the intracellular structures and metabolism. The second reflects the reaction of the body as a whole to the radiation effect [3].

In this work we shall consider local irradiation of the tissue, assuming that the reaction of the tissue to irradiation is of a local character. This means that the state of the irradiated area is determined solely by the processes occurring in this area.

Hence, our investigation is concerned with a closed population of tissue cells. In normal conditions this population can be considered as homogenous and its state described by the following differential equation (quoted in [1] and [4]):

$$\frac{dp}{dt} = \left(\Omega_0 - \omega_0 p - \frac{1}{\tau_0}\right)p. \tag{1}$$

Here $p(t)$ is the number of cells per unit volume tissue; $k_0(p) = \Omega_0 - \omega_0 p$ is the probability of division of the cell per unit time, $1/\tau_0$ (where $\tau_0$ is the mean life span of the cell) is the probability of its death.

The structure of the multiplication coefficient $k_0(p)$ reflects the fact that probability of division per unit time is not constant for a given type of cell and diminishes with an increase in $p(t)$ because of the interaction of the cells during their vital activity.

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It is obvious that the numerical value of $\Omega_0$ is determined by the nature and state of the multiplication apparatus of the cell and $\omega_0$ by the intensity of cell metabolism.

After irradiation of tissue, cells appear in it damaged to varying degrees by irradiation and the cell population of the tissue becomes inhomogenous. Its state is now described by the set of equations [4]

$$\frac{dp_i}{dt} = \left(\Omega_i - \sum_k \omega_k^i p_k - \frac{1}{\tau_i}\right) p_i - \sum_k p_i a_{ik} + \sum_k p_i a_{ki}. \tag{2}$$

Here $p_{ik}$ is the number of cells of group $i$ per unit volume irradiated tissue, $k = \Omega_1 - \sum_{k'} \omega_k^i p_k a_{ik}$ is the probability of division of the cell of the $i$ group per unit time, $1/\tau_i$ the probability of its death, $a_{ik}$ the probability of transition of the cell from the $i$ group to the $k$ group as the result of irradiation damage to the cells and their recovery. Summation occurs for all the cell groups corresponding to the differing degree of their radiation damage.

If we assume that the number of cells damaged by irradiation per unit time is proportional to the radiation does rate then the probability of the transition $a_{ik}$ can be represented in the form

$$a_{ik} = a_{ik}^0 + a_{ik}^1; \quad a_{ik}^1 = a_{ik} D(t) \tag{3}$$

where $a_{ik}^0$ is the regeneration probability of the transition, $a_{ik}^1$ the radiation probability of transition, $D(t)$ the dose rate, $a_{ik}$ biological effectiveness of irradiation in relation to the transition $i \longrightarrow k$.

Summating the equations of the sytem (2) we obtaine the equation for the total number of cells per unit volume of irradiated tissue $p(t)$.

$$\frac{dp}{dt} = \left(\Omega - \frac{1}{\tau}\right) p - \omega p^2, \tag{4}$$

where the values

$$\Omega - \frac{1}{\tau} = \sum_i \left(\Omega_i - \frac{1}{\tau_i}\right) \cdot \frac{p_i}{p}; \quad \omega = \sum_i \sum_k \omega_k^i \frac{p_i}{p} \cdot \frac{p_k}{p} \tag{4a}$$

are the average for all groups of cells of the population. Information on the numerical values of $\Omega$, $\tau_i$ and $\omega_{ik}$ can be obtained from analysis of the findings of the nature of the radiation damage to the intracellular structures and metabolism. On irradiation with sufficiently low doses only the most radiosensitive processes are affected, the most vitally important of which are mitosis, synthesis of DNA and for certain tissues, oxidative phosphorylation [5]. Damage to the mitotic apparatus or DNA synthesis does not lead to change in the viability of the cell but merely suppresses its capacity for division. Therefore, the intensity of intracellular metabolism and the growth rate of the cell change slightly. This phenomenon was observed for example, by Forrsberg [5] on irradiation of ascites tumour cells. In this case $\omega_{ik} \approx \omega_0$, $\tau_i \approx \tau^0$ and only $\Omega_i$ appreciably differ from $\Omega_0$. Since oxidative phosphorylation is one of the basic proceses supplying energy for the vital activity of the cell, then its impairment leads to a sharp change in the viability of the cell and intensity of intracellular metabolism. In this case, $\Omega_i <$

$< \Omega_0$, $\omega_k^i < \omega_0$ and $\tau_i < \tau_0$. A similar picture is observed in the tissues with radioresistant oxidative phosphorylation on irradiation with large doses when these less radiosensitive links of intracellular metabolism start to suffer.

We shall assume at first that in the irradiated tissue there exist only two types of cells; cells which have avoided radiation damage ($i = 0$) and, cells the mitotic activity of which is completely suppressed ($i = 1$, $k_1 = 0$).

In this case the set of equations (2) assumes the following form:

$$\begin{cases} \dfrac{dp_0}{dt} = \left(\Omega_0 - \dfrac{1}{\tau_0} - \omega_0^0 p_0 - \omega_1^0 p_1\right) p_0 + \alpha_{10}^0 p_1 - \alpha_{01} D(t)\, p_0; \quad p_0(0) = p(0) \\ \dfrac{dp_1}{dt} = \alpha_{01} D(t) p_0 - \left(\alpha_0^0 + \dfrac{1}{\tau_1}\right) p_1; \quad p_1(0) = 0. \end{cases} \tag{5}$$

Solution of the system (5) for the arbitrary function $D(t)$ may be obtained only by numerical methods, however, a solution reflecting the reaction of the tissue to pulsed radiation the duration of which is much less than the least of the time characteristics of the tissue may be obtained analytically. Bearing in mind that during irradiation, the intensity of the radiation damage is much greater than the intensity of cell division and intracellular repair and choosing zero time as the moment of termination of radiation we can in this case rewrite the system (5) in the form

$$\begin{aligned} \frac{dp_0}{dt} &= \left(\Omega_0 - \frac{1}{\tau_0} - \omega_0^0 p_0 - \omega_1^0 p_1\right) p_0 + \alpha_{10}^0 p_1; \quad p_0(0) = p(0)\, e^{-\alpha_{01}\Delta} \\ \frac{dp_1}{dt} &= -\left(\alpha_{10}^0 + \frac{1}{\tau_1}\right) p_1; \quad p_1(0) = p(0)\,(1 - e^{-\alpha_{01}\Delta}) \end{aligned} \tag{6}$$

where $\Delta = \int_0^a D(t)dt$ is the integral dose received by the tissue during irradiation. It is not difficult to conclude from an analysis of the structure of system (6) that the process of repair of radiation damage to tissue is based on two mechanisms; the mechanism of intracellular repair, the intensity of which $\alpha_{10}^0 p_1$ is derermined by the repair processes within the radiation damaged cells and the mechanism of tissue regeneration, the intensity of which $\left(k_0 - \dfrac{1}{\tau_0}\right) p_0$ is determined by the intensity of multiplication of the undamaged cells. If the condition:

$$\alpha_{10}^0 p_1 \geqslant \left(k_0 - \frac{1}{\tau_0}\right) p_0 \tag{7}$$

is satisfied, then the main contribution to restoration of radiation damage of tissue is made by the processes of intracellular repair. Solution of the system (6) in this case is given by expressions:

$$\begin{aligned} &p_1(t) = p_1(0) e^{-\frac{t}{\tau}}, \quad \frac{1}{\tau} = \alpha_{10}^0 + \frac{1}{\tau_1}, \\ &p_0(t) = p_0(0) + \alpha_{10}^0 \tau p_1(0)\,(1 - e^{-\frac{t}{\tau}}). \end{aligned} \tag{8}$$

VOL. 5 1960

If the condition

$$\left(k_0 - \frac{1}{\tau_0}\right) p_0 \gg \alpha_{10}^0 p_1 \tag{9}$$

is satisfied, then tissue regeneration predominates. The system (6) has in this case the following solution:

$$p_1(t) = p_1(0)\, e^{-\frac{1}{\tau}}, \quad \frac{1}{\tau} = \alpha_{10}^0 + \frac{1}{\tau_1},$$

$$p_0(t) = p_0(0) \cdot \frac{e^{\int_0^t \left(\Omega_0 - \frac{1}{\tau_0} - \omega_1^0 p_1\right) dt}}{1 + p_0(0)\,\omega \int_0^t e^{\int_0^t \left(\Omega_0 - \frac{1}{\tau_0} - \omega_1^0 p_1\right) dt} dt}. \tag{10}$$

It is easy to establish from an analysis of expressions (7) and (9) that the mechanism of restoration depends not only on the nature of the tissue but on the degree of its damage. With sufficiently minor injuries to the tissue when the condition

$$\frac{p_1(0)}{p_0(0)} \leqslant \frac{k_0(0) - \frac{1}{\tau_0}}{\alpha_{10}^0} \tag{11}$$

is satisfied, the mechanism of restoration is primarily regeneration. If the damage is sufficiently great and the condition

$$\frac{p_1(0)}{p_0(0)} \leqslant \frac{k_0(0) - \frac{1}{\tau_0}}{\alpha_{10}^0} \tag{12}$$

is satisfied, then in the initial period of restoration, the predominant role will be played by the processes of intracellular repair. With the development of the restorative process the mechanism of tissue regeneration comes into play and becomes predominant in the period of completion of restoration.

In conclusion, it is necessary to say a few words on the possibility of checking the above statements experimentally. A direct count of number of damaged and undamaged cells is impracticable because it is often impossible to distinguish visually the cells damaged by radiation from the undamaged ones and the experimenter can count only the total number of cells per unit volume of tissue. In our approximation the essence of radiation damage lies in the complete suppression of the ability of the cell to divide and in curtailment of its life span. Proceeding from this, from the content of the dividing cells in the irradiated tissue we may judge which part of the cells of the tissue is damaged. In fact, drawing up a balance of the dividing cells from the number of mitoses per unit volume of tissue $M(t)$ it is quite easy to obtain the following equation:

$$\frac{dM}{dt} = k_0 p_0 - \frac{M}{\tau_M} \tag{13}$$

(where $\tau_M$ is the duration of mitosis) the solution of which is the expression

$$M(t) = e^{-\frac{t}{\tau_M}} \left( M(0) + \int_0^t k_0 p_0 e^{\frac{t}{\tau_M}} dt \right). \tag{14}$$

However, analysis of the experimental data on radiation impairment of cell division shows that there exist phenomena which do not fit into the confines of the two-group approximations considered above. The question is one of the so-called pathological mitosis corresponding to partial impairement of the ability of the cell to divide. It can be allowed for within the framework of a three-group approximation. We shall bring together in the first group ($i = 0$) as before, all the undamaged cells, into the second ($i = 1$) the cells the mitotic activity of which has been completely suppressed and finally, the third group ($i = 2$) cells with partially impaired ability to divide, giving rise to various forms of pathological mitosis. Considering as before brief pulsed radiation and choosing as zero time the termination of radiation we can write the system (2) in the form:

$$\begin{cases} \dfrac{dp_0}{dt} = \left( \Omega_0 - \dfrac{1}{\tau_0} - \sum\limits_{k=0}^{2} \omega_k^0 p_k \right) p_0 + a_{10}^0 p_1 + a_{20}^0 p_2 \\ \dfrac{dp_1}{dt} = -\left( a_{10}^0 + a_{12}^0 + \dfrac{1}{\tau_1} \right) p_1 \\ \dfrac{dp_2}{dt} = \left( \Omega_2 - \dfrac{1}{\tau_2} \sum\limits_{k=0}^{2} \omega_k^2 p_k \right) p_2 + a_{12}^0 p_1 - a_{20}^0 p_2. \end{cases} \tag{15}$$

Considering that during irradiation, the intensity of irradiation damage is much greater than the intensity of cell division or intracellular regeneration and solving system (2) for this hypothesis with $i = 0$, 1 and 2 for the space of time coinciding with the period of irradiation, it is easy to obtain expressions for the initial condition of system (15):

$$\begin{cases} p_0(0) = p(0)\, e^{-(a_{01}+a_{02})\Delta} \\ p_1(0) = p(0) \left\{ 1 - \dfrac{a_{02}\, e^{-a_{21}\Delta}}{a_{01} + a_{02} - a_{21}} + \dfrac{a_{02} \cdot a_{21} - a_{01}(a_{01} + a_{02} - a_{21})}{(a_{01} + a_{02} - a_{21})(a_{01} + a_{02})} e^{-(a_{01}+a_{02})\Delta} \right\} \\ p_2(0) = p(0) \cdot \dfrac{a_{02}}{a_{01} + a_{02} - a_{21}} \cdot \left\{ e^{-a_{21}\Delta} - e^{-(a_{01}+a_{02})\Delta} \right\}. \end{cases} \tag{16}$$

The general solution of the system (15) may be obtained only by numerical methods. However, for certain particular cases the approximated solution can be determined analytically. It is easy to see that for the irradiated tissue the condition

$$p_2 \ll p_0 + p_1 \tag{17}$$

is usually fulfilled.

VOL. 5 1960

In fact, on irradiation with low doses, the number of pathological mitoses is insignificant and $p_0 \gg p_2$; if the tissue is irradiated with a large dose and most of the cells are damaged by irradiation, then $p_1 \gg p_2$. Therefore, for certain tissues the conditions

$$\omega_0^1 p_0 + \omega_1^1 p_1 \geqslant \omega_1^1 p_2$$
$$\left(k_0 - \frac{1}{\tau_0}\right) p_0 + \alpha_{10}^0 p_1 \geqslant \alpha_{20}^0 p_2 \tag{18}$$

may be fulfilled, implying that the effect of the partially damaged cells on the intensity of cell division and the contribution of their restoration to the increase in the number of normal cells is negligible because of their low number. Disregarding the small values in equations (15) we obtain for $p_0$ and $p_1$ the equaations of a two-group approximation investigated in detail above, the only difference being that now

$$\frac{1}{\tau} = \alpha_{10}^0 + \alpha_{12}^0 + \frac{1}{\tau_1} \tag{19}$$

and the initial values are determined by expressions (16). Solving the third equation of system (15) we obtain the expression for $p_2(t)$:

$$p_2(t) = e^{-\int_0^t \left(\Omega_2 - \frac{1}{\tau_2} - \alpha_{20}^0 - \sum_{k=0}^{1} \omega_k^2 p_k\right) dt} \times$$
$$\times \left\{ p_2(0) + \alpha_{12} \int_0^t p_1(t) e^{\int_0^t \left(\Omega_2 - \frac{1}{\tau_2} - \alpha_{20}^0 - \sum_{k=0}^{1} \omega_k^2 p_k\right) dt} \cdot dt \right\}. \tag{20}$$

As can be readily seen change in the value $p_2(t)$ is the result of the preponderance of multiplication of the partially damaged cells over their death or repair. The probability of the two latter processes does not depend on time, though the probability of division increases with development of the restorative processes in view of the fact that $\omega_0^1 \geqslant \geqslant \omega_1^1$. In a certain period of time directly after irratiadion multiplication of partially damaged cells may predominate over death and repair and $p_2(t)$ may increase for a certain time but because $\Omega_0 - \frac{1}{\tau_0} > \Omega_2 - \frac{1}{\tau_2}$ only the more viable normal cells ultimately remain in the tissue and $p_2(t) \to 0$ for $t \to \infty$.

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# METHODS AND INSTRUMENTS

## CYTOMETRIC ANALYSIS OF BLOOD BY ELECTRONIC METHODS*

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L. B. KAMINIR and L. L. LITINSKAYA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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CYTOMETRIC analysis of the blood is widely used for the diagnosis and prophylaxis of diseases and also in various biological investigations. It consists in visual determination by means of a microscope and a counting chamber (haemocytometer) of the number of blood cells of given types in a specially prepared sample. The results of measurements are calculated per 1 mm³ blood. Such a method of analysis has serious shortcomings: low accuracy, laboriousness and the considerable time required to make the measurements. We shall deal with these shortcomings in greater detail.

The visual count of the blood cells causes comparatively rapid fatigue of the laboratory worker. Because of this, the number of cells counted in the usual conditions is small and amounts for example, on counting of erythrocytes, to about 500. If we compare this figure with the number of erythrocytes in 1 mm³ blood ($5 \times 10^6$) and take into account the factor of chance in the distribution of the erythrocytes in the sample, then it becomes obvious that there is high degree of statistical error in performing cytometry. For example, the statistical error, i.e. the standard deviation ($\sigma$) on counting the number of erythrocytes is about 4 per cent [1, 2]. This means that the degree of error for two-thirds of the counts is within these limits, 95 per cent of the counts have an error not exceeding $\pm 8$ per cent and 5 per cent have an error exceeding $\pm 8$ per cent. Consequently, it may be considered that the statistical error in determining the number of erythrocytes is about $\pm 8$ per cent ($2\sigma$).

The total error of measurement exceeds the statistical one because of the errors arising in the count of the formal elements in the chamber for the following reasons:

(1) errors due of the unequal distribution of the formal elements in the counting chamber; their concentration increases towards the side opposite to where the sample was introduced;

(2) errors because of the fact that the same particle may be counted several times of a certain portion of the particules may be omitted in the count;

(3) errors because of the imprecision in diluting the blood with saline and bacause of the lack of agreement between the true volume of the haemocytometer and the rated value, these, in turn, determine the error in fixing the relation between the number of elements counted in the sample and the number of elements actually contained in 1 mm³ blood; and

(4) errors because of evaporation of the saline in the counting chamber which leads to an increase in the concentration as against the actual one; included here is the error arising from haemolysis of the erythrocytes, which somewhat reduces their true concentration,

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In view of the above remarks it may be considered that the actual error of the red cell count is about ±10 per cent and in a number of cases exceeds this value.

The statistical error of the white cell count is greater than for the red cell count, since the concentration of leucocytes in the blood is much smaller. Because of this, the factor of random distribution of leucocytes in the samples taken is increased. Its increase also results from the leucocyte capacity for amoeboid movement so that their concentration in different parts of the blood system varies somewhat. Usually the statistical error in the leucocyte count is ±7 per cent [3] and the actual error about 20–30 per cent.

It is obvious that the high error in the visual count of the formal blood elements in a number of cases prevents detection of changes actually occurring in the body.

It takes the experienced laboratory worker 10 to 15 min to carry out the analysis even with such low accuracy. At the present level of medical services and research work in therapeutic and research institutes, a large number of blood analyses are made so that thousands of laboratory workers are engaged in this work. On the other hand, possibly in cases of sudden intensive radiation (for example, on disturbance of the normal operations of atomic plant — "its warming up") the available complement of laboratory workers may prove entirely inadequate to make a rapid determination of the degree of radiation injury to the attendants.

The defects in visual blood analysis considered above unequivocally point to the desirability of automating analysis. The advantages of automation lie in the possibility of obtaining high accuracy (the factor of fatiguability does not arise for the instrument and therefore, on counting a large number of particles, the basic, i.e. statistical error, can be reduced to a minimum) and the possibility of sharp increase in the speed of carrying out the analysis and finally, in replacing the fatiguing work of the laboratory workers by the work of an automaton.

To illustrate this, and running ahead a little, we would point out that a count of the number of thrombocytes made with high accuracy by means of one of the electronic methods makes it possible within only 30 min of radiation with a dose of 10 r to establish reliably changes in the blood associated with this factor. The time taken to carry out one analysis is about 1 min [4].

The advantages of automatic analysis stand out even more clearly in the case of qualitative morphological analysis, for example, erythrometry. It is known that change in the size of erythrocytes serves as an indirect but important indication of the work of a number of human organs (for example, the liver), indication of the state of the entire organism, the function of the haemopoietic system etc. It is desirable to determine in this case not only the mean values of the size of the erythrocytes but also, to know the curve of size distribution of the erythrocytes. It is obvious that measurement of hundreds of erythrocytes with an ocular-micrometer is an extremely laborious operation taking up much time and therefore carried out only in exceptional cases. Automation of this method immediately transforms it into a method for mass investigation [5].

The active drive for automation of the processes of blood analysis started about 10 years ago. At first, the main direction of automation was along the lines of modelling the work of the laboratory assistant. The blood specimen for analysis was placed in front of the microscope. Instead of the eye of the laboratory assistant, the transmitting tube of television device was fixed to the ocular of the microscope serving to convert the optical picture into a totality of electrical signals. The problem of decoding the pulses obtained was solved by an electronic method using valve circuits [6–10]. Such devices were raised to the level of complete technical perfection in a number of cases (for example [9]) but apparently, were not widely adopted. Part of the reason for this is that the difficulties of decoding the pulses purely by electronic means leads to the need for limiting the problem solely to a count of erythrocytes or leucocytes. A differential leucocyte count has not been carried out by these devices. Further, They are very expensive and complex and require comparatively skilled servicing of the apparatus by an electronic specialist.

After the appearance of these contrivances the efforts of scientists were directed at solving two main problems; simplification of the methods of automatic analysis and extension of it possibilities. The results of prolonged work were comparatively simple devices for determining the number

of particles and their distribution according to size [1]. These are based not on television scanning of an immobile preparation but on movement of the particles in front of an optical or some other system [11, 12]. Such devices turned out to be much simpler and cheaper than analysers containing a television unit. However, the possibilities of these systems are limited.

Extension of the potentialities of automatic analysis requires consideration of many factors. Since sufficient information on the particles may be obtained by visual observation, there has been a return to the method of television scanning with subsequent analysis of the fine structure of the signals by systems making use of the latest achievements in computer techniques [13]. The comparatively great complexity of these devices will probably be offset by their possibilities and also the high accuracy of the work.

In order to facilitate the review of existing systems of cytometric analysis of blood we shall divide them into two major groups; the first includes systems distinguishing the blood elements from their optical properties, the second from their electrical properties. We shall start the review with the second and less numerous group.

VOL. 5 1960

## SYSTEMS DISTINGUISHING BLOOD ELEMENTS ACCORDING TO THEIR ELECTRICAL PROPERTIES

It was pointed out above that the trend towards simplification of automatic analysis of blood has led to the design of devices distinguishing the blood elements by a small number of criteria but having considerable simplicity and high accuracy. One of the properties utilized in such devices is that of "electrical contrast". It is based on the fact that the electrical resistance of the blood cells is much greater than that of the saline in which these cells are suspended. The utilization of this property has made possible mass production of instruments, for example, the "Coulter Counter" (Coulter Electronics, U.S.A.) and the "Celloscope" (A. B. Lars Ljungberg & Co., Sweden).

The idea of the instrument is as follows [1]. A suspension of particles in saline is forced through a small aperture A (diameter $\sim 0{\cdot}1$ mm, length $\sim 0{\cdot}15$ mm) connecting two electrically insulated vessels filled with saline (Fig. 1). Under the action of a source of the electrical voltage ($U_{source}$) between the electrodes E immersed in saline there is flow of a current the value of which depends on the resistance of the entire circuit. The resistance of the saline is low. If a blood cell with high resistance enters aperture then because of the reduction in the current-carrying capacity of the aperture, which in the main determines the resistance of the entire electrical circuit, the latter is appreciably increased.

The blood is added to the saline to guive a concentration of about $(20–80)\times 10^3$ cells per 0·5 ml saline. The suspension of particles is driven through aperture A by means of a small vacuum pump H (time of passage of particles through aperture about 10–20 ($\mu$sec).

The strength of the current charges at the moment of passage of the particles through the aperture and a voltage pulse is formed across the resistance, the value of which depends on the dimensions of

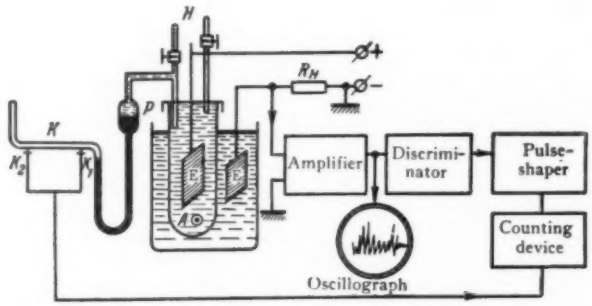


FIG. 1. Block diagram of automatic counter of micro-particles utilizing phenomenon of "electrical contrast".

the blood cell. This pulse is amplified and enters the counter. By setting the threshold of the voltage discriminator preceding the counter at a definite level it is possible to make a count of the cells with dimensions greater than or equal to a certain value. To simplify this operation a visual indicator of pulse size is used (an oscilloscope tube).

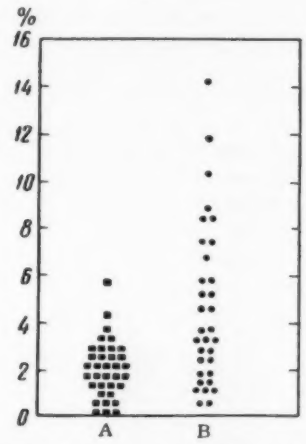


FIG. 2. Assessment of results of measurements of erythrocytes estimated by automatic counter (A) and in haemocytometer (B). Ordinates give percentage difference between results of counts in two identical preparations.

The number of particles with dimensions lying in a definite interval is found as the difference between the number of particles exceeding both levels taken separately.

The rate of passage of the suspension (the rate of count) is determined by the degree of vacuum used. The constancy of volume of solution in which the number of blood elements is determined is ensured by the fact that mercury from P is expelled into the elbow K and successively closes contacts $K_1$ and $K_2$ thereby determining the start and end of the count.

The principal merit of this method is that it makes possible determination not only of the diameters of the particles but their volume which in a number of cases is of considerable interest [14]. Applying conventional methods of analysis to find the volume of the cells it is necessary to determine their diameters, the number of cells and the mean thickness of the cells (centrifugation is used for the latter) which leads to a low accuracy of measurement.

The factors determining the accuracy of the method of electrical contrast are considered in papers [1, 15] and chiefly in [11].

The following factors affect the error in the count of the blood elements of given size:

1) the accuracy of measurement of the volume of solution (here the error may be up to $\pm 0{\cdot}1$ per cent);

2) the error arising from the fact that not one but several particles may pass simultaneously through the aperture, this error is lower the lower the ratio of the size of the aperture to the size of the particles under analysis and the lower the concentration of the solution — it may be evaluated by means of the theory of probability;

3) the error due to partial obstruction of the aperture causing changes in the calibration of the discriminator;

4) the error arising from obstruction by the saline (to reduce which the saline is first passed through glass filters), included in this category are errors which may arise because of the presence of bubbles of air entering the aperture and also particles from the surface of the electrodes, this can be avoided by the using platinum electrodes (16); and

5) the error due to imprecision of dilution of blood with saline, an error, as pointed out before, present for all methods, but which can be reduced by automating the dilution of blood in saline.

Of interest are the measurements made for the purpose of comparing the accuracy of an instrument using the method of electrical contrast with the accuracy of measurements from a visual count in a counting chamber [11]. In each case 35 control measurements were made. Their results are shown in Fig. 2. The mean error of the instrument may be considered as about 2 per cent. Only 2 measurements with the instrument gave an error of more than 4 per cent, one of them a maximum error somewhat less than 6 per cent. In contrast, with measurements in the counting chamber an error exceeding 4 per cent occurred in 12 cases and the maximum error was 18 per cent. As an example, we give some data on the instrument known as the Celloscope [16, 17]. Its exterior is as shown in Fig. 3.

By means of the Celloscope it is possible to determine the number of erythrocytes, leucocytes and thrombocytes and also distribution by size of erythrocytes and leucocytes (Price-Jones curves). For analysis of erythrocytes a dilution of blood of 1:80,000 is used, for analysis of leucocytes 1:800 and in acute leucocytosis a dilution of 1:8000.

When investigating leucocytes a reagent destroying the erythrocytes is added to the solution. To reduce errors in the count due to the presence of erythrocyte ghosts fullest possible haemolysis of erythrocytes is achieved by increasing the time of haemolysis (minimum time 15 min).

VOL. 5 1960

FIG. 3. Exterior of Celloscope.

In the case of determining the distribution of particles according to size the solvent used is not ordinary saline, since its osmotic pressure would lead to a change in the shape of the test particles, but a special buffer solution (250 ml 0·9% NaCl, 45 ml phosphate buffer, pH 7·55, 5 ml neutral 35% solution of formaldehyde).

Attempts are now being made to apply the Celloscope not only for blood analysis but also for counting the number of bacteria, the cells of certain tissues, etc. The size range of particles measured by the Celloscope is from 1 to 200 $\mu$. To ensure the possibility of such a wide range the vessels used in the instrument have calibrated apertures (diameter of the apertures must be 2–4 times greater than the average size of the particles submitted to analysis). The Celloscope is compact and ensures good accuracy of measurement (error less than 2·5 per cent) and very fast action (500 preparations in 8 hr).

To sum up, it may be stated that the method utilizing the property of electrical contrast is quite simple, effective and cheap. However, the prospects of this method are limited by the fact that it allows discrimination of the blood elements solely according to their dimensional gradations.

## SYSTEMS DISTINGUISHING THE BLOOD ELEMENTS ACCORDING TO THEIR OPTICAL PROPERTIES

Such systems are now very numerous and are being developed along two lines. The first applies to the simpler and cheaper systems in which the preparation to be analysed (dried blood smear on a slide or suspension of blood in a capillary tube or haemocytometer) is moved in relation to the optical analyser—source of light and the light sensitive element or, on the contrary, the optical analyser is moved in relation to a fixed object. Scanning of a stationary preparation on a fixed optical analyser is also possible by means of a mobile optical system — rotating prisms for example [18].

The second line of development includes systems in which the test preparation is submitted to successive analysis by means of one of the methods used in television, i.e. scanned by an element the dimensions of which are less than those of the particles being measured.

The work of the analysers belonging to the first group is based on the following principle.

When the blood cell is between the source of light and the light sensitive element (usually a photomultiplier) the intensity of the light flux is changed.

Since the field of vision of the photomultiplier is confined by the diaphragm and the blood particles move in relation to the photomultiplier, electrical pulses corresponding to the moving particles are obtained at its output. Given constant speed of movement of the particles, the duration of the pulses will be proportional to the dimensions of the particles in the plane of their movement in front of the photomultiplier. This makes possible analysis of the dimensions of the particles.

In all these cases the relative displacement of particle and photocell is fundamental. Therefore, we shall confine ourselves to consideration only of systems with mechanical movement of the test preparations in relation to the optical analyser.

For convenience, we shall consider separately analysers in which the blood suspension moves along a transparent capillary tube.

*Systems with mechanical movement of test preparation.* The movement of a dried blood preparation or heamocytometer is usually carried out by mechanical systems set in motion by a small motor. Thus, for example, in the analysers in references [12 and 19] the stage of the microscope is slowly moved towards one side in a longitudinal direction and moves much more rapidly forward and backward in a direction perpendicular to the first. Thus, the blood preparation is scanned by a curve approximating to a sinusoid.

It is obvious that in this case not all the blood cells present in the preparation but only part of them will be counted. The total number of cells may be obtained by multiplying those actually counted by a coefficient depending on the volume of blood being inspected (equal to the depth of the chamber and the area inspected) and the degree of dilution of the blood.

Sinusoidal scanning of the sample may be replaced by straight line scanning, i.e. a considerably simplified mechanical system confined to the linear movement of the blood preparation [20]. In this case, the error of the count does not exceed 2–3 per cent. A repeat count for different areas and in different directions, for which provision is made in the analyser [20], is possible and averaging the results of the counts appreciably increase the accuracy of the instrument.

Both the first and second analysers require preliminary determination of the optimum level of discrimination. In the latter this depends on the magnification of the microscope, luminosity, focusing, particle size, type and systems of photomultiplier, etc. It is obvious that the correct setting of the discrimination threshold will determine whether the instrument distinguishes the blood cell to be counted from any random inclusion which may enter the preparation or from a pulse caused by change in voltage of the supply source or any other cause. However, it must be borne in mind that for any one discrimination level, some of the particles intersected by the boundaries of the field of vision of the photomultiplier will not be counted.

To lessen the effect of the discrimination level on the results of the measurements and prevent losses in counting due to boundary particles it has been proposed that a double count be made of the number of elements for different dimensions of the scanning area (different slit width) [12, 21].

Then the difference between the instrument readings obtained in both cases will, on the one hand

depend considerably less on the discrimination threshold and, on the other, the counting error due to the presence of boundary particles will be reduced to a minimum.

In fact, if the number of blood cells actually present in the area being scanned in the first and second case are denoted respectively as $n_1$ and $n_2$ and the particles counted by the instrument as $N_1$ and $N_2$ and the particles falsely counted as $\Delta N_1$ and $\Delta N_2$ (the latter may be positive or negative) then $n_1-n_2 = (N_1+\Delta N_1)-(N_2+\Delta N_2) = N_1-N_2+(\Delta N_1-\Delta N_2)$.

If the spurious readings are determined by interference in the electron-optical part of the circuit and the particles located at the boundaries of the field of vision not taken into account one can with sufficient accuracy consider that $\Delta N_1 \approx \Delta N_2$.

In this case $n_1-n_2 \approx N_1-N_2$.

In reality, the picture is more complex. The accuracy of the count is influenced by the choice of area of the slits, the constancy of the illumination and scanning velocity, the sensitivity of the light recording system, presence of adjacent particles, etc. The influence of these factors has been analysed in detail in paper [18].

Movement of the blood suspension in front of the electron-optical device is also carried out in the analyser in paper [22]. However, here determination is made not of the number of blood elements in a given volume as in the preceding cases, but the reciprocal, i.e. the average volume of solution per cell. The optical system of the analyser is so designed that a light flux passing through a very small zone of the suspension (an ordinary microscope is used with a dark field condenser and a limiting diaphragm), impinges on the photocathode of the multiplier.

With suitable dilution most of the time during which the given volume of suspension is being examined it is free of blood particles and a small dark current flows (depending on the load of the photomultiplier).

When a blood cell passes through the volume being examined, the light scatteerd by it enters the objective of the microscope and causes an increase in the current of the photomultiplier.

The duration of the pulse so formed should correspond to the time the particle is in the volume under examination.

If $v_m$ is the mean volume of solution per particle, $v$ the volume being examined, $t$ the time of measurement and $t_t$ the total duration of the pulses determined by the passage of the blood particles, then

$$v_m = \frac{vt}{t_t}$$

Since the pulse shaper ensures constancy of amplitude of the pulses, then the mean current at the output of the circuit is

$$I_m = K\frac{t_t}{t}$$

Here, $K$ is the coefficient of proportionality depending on the parameters of the pulse shaper and the sensitivity of the instrument. The instrument may be graduated in units of the concentration of particles.

Replacement of a discrete count by receipt of continuous information somewhat reduces the accuracy of the instrument. The advantage of the method is the absence of dependence of the indications on the speed of movement of the sample, simplicity of work with the instrument (it is not necessary to select a discrimination threshold), etc. We would note that there are now industrial models of analysers using the principle of mechanical movement of the blood preparation (for example, the analysers issued by the Iarrell-Ash Company and Casella (Electronics) Limited [17, 19].

*Systems with movement of blood in a capillary.* The idea of analysing blood whilst moving in a capillary arose from the study of blood flow in the animal organism. In particular, the method considered below was used to count the blood elements of a living frog [23]. If we pass a blood suspension in saline through a capillary placed in front of an optical system and a photosensitive element in such a way that only one particle enters the field of vision, then the counter set up behind the photocell

VOL. 5 1960

will record the number of particles passing through the capillary. The first steps in this direction were taken as early as 1934 [18].

A number of difficulties arise in applying this method. The rate of passage of the blood suspension through the capillary $V$ is determined by the relation

$$V = K\frac{D^2}{\eta}$$

here $K$ is a coefficient of proportionality, $D$ is the diameter of capillary and $\eta$ is the viscosity coefficient.

To increase the speed of the instrument it is desirable to increase the diameter of the capillary $D$. However, this results in an increase in the count losses because of the simultaneous passage of several particles along the capillary and difficulty in focusing the microscope because of the low degree of definition of the capillary.

Another difficulty is that the speed of the particles moving in the capillary is not constant and changes from zero at the capillary walls to maximum at the centre. The inconstancy of the velocity prevents analysis of the particles according to size, since the duration of the pulse taken from the photomultiplier is a function not only of the diameter of the particle, but of its location in the capillary. This difficulty has been successfully solved by designing a capillary with liquid walls [24, 25].

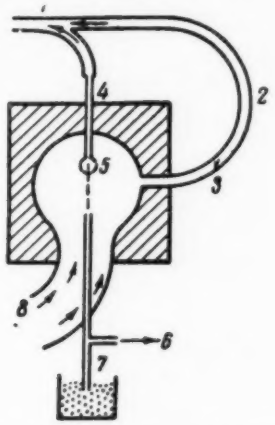


FIG. 4. Arrangement of counting chamber: 1 and 2, sections of pump for circulation; 3, valve; 4, upper capillary; 5, observation zone; 6, to micro-pump; 7, lower capillary; 8, water flow produced by pump.

Fig. 4 shows a diagram of the counting chamber of the instrument. The blood suspension rises up capillary 7 and is expelled into the counting chamber by a pulsating micropump. A circulating pump forms in the counting chamber an ascending stream of water with a diameter of 20 $\mu$ which entrains the microparticles carrying them in front of the recording instrument. The analyser gives an erythrocyte count in 10 sec with an accuracy of $\pm 2{\cdot}1$ per cent.

The leucocyte count, in principle, does not differ from that for the erythrocytes and is carried out in the same time with an accuracy of $\pm 9$ per cent. To increase the accuracy the number of particles being counted may be increased.

On the basis of this principle the firm of Evans Electroselenium Limited has organized mass production of blood analysers. A later instrument produced by this firm was converted for thrombocyte counts which made it possible to use for the rapid determination of the presence of radiation injury [4, 17].

The accuracy ensured by this modification of the instrument with a 10-fold count was as follows; for erythrocytes $\pm 0{\cdot}37$ per cent, leucocytes $\pm 3{\cdot}52$ per cent, thrombocytes $\pm 4{\cdot}68$ per cent.

The rate of investigation is 100 preparations per hour. The instrument can also be used for measuring the size distribution of particles. Another of its advantages is that deformation of the dimensions of the particles is considerably less than in investigations of dried smears.

On the basis of the capillary method an instrument has also been proposed for the simultaneous count of erythrocytes and leucocytes [26] consisting of a tube with a capillary construction where the passes, a semi-transparent mirror, light filters and two photocells (Fig. 5).

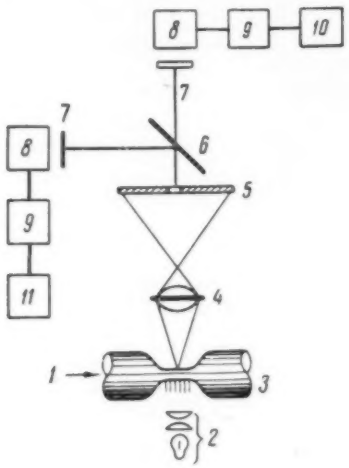


FIG. 5. Block diagram of capillary analyser of micro-particles: 1, blood; 2, illuminator; 3, capillary; 4, magnifying lens; 5, diaphragm with slit; 6, semi-transparent mirror; 7, light filter; 8, photomultiplier; 9, amplifier; 10, erythrocyte counter; 11, leucocyte counter.

Staining of blood results in the leucocytes and erythrocytes assuming different colours, then by means of two light filters the optical signals produced on passage of the leucocytes and erythrocytes in front of the light source are differentiated. These signals enter the corresponding photomultipliers and pulse counters.

It is desirable to limit the field of vision of the photomultiplier to the plane of the enlarged image of the narrow part of the capillary.

Owing to a number of shortcomings (difficulties in focusing, inconstancy of speed of movement of particles along the section of the capillary, high level of interference due to the impossibility of using a dark image field since the walls of the capillary diffract the rays, etc.) this instrument has not found practical application.

*Systems utilizing television methods of scanning.* As stated above, the use of television methods requires complex and cumbersome apparatus which is not justified for counting the number of particles and determining the distribution of particle size.

The methods of television scanning can be divided into two groups:

(1) the preparation under test is scanned (method of scanning beam),

(2) an image of the test preparation is scanned.

In our review there is no point in considering in detail methods of television analysis since they have been adequately dealt with and systematized in the literature [27, 28].

It must be stated that the problem of the complete automation of the process of blood analysis including determination of the leucocyte differential can probably be solved only on the basis of application of television methods of inspection of the preparation with subsequent analysis of the signals from each elementary particle by means of computers [13, 29].

VOL. 5 1960

The volume of information and consequently the volume of the memory of the computer necessary for differential determination of all the formal elements of the blood is very high. The automatic analyser must distinguish not only the dimensions of the particles but their shape and colour, granularity, position and shape of nucleus, etc. [30].

The complexity and consequently, the cost of such an instrument increases with increase in the number of criteria underlying differentiation of the blood elements and the reliability of the instrument is thereby substantially reduced.

Hence, the need arises for working out a classification of the formal elements in a clear and suitable form for automatic analysis and, on the other hand, raising the reliability of complex circuits (one of the methods of increasing reliability is the application of ferrite magnetic elements for computer devices).

An automatic blood analyser of the type described in [13] may ensure very high productivity and accuracy of analysis of blood which will considerably improve the diagnosis of diseases from the blood pictures by bringing out details and interrelations in the living body previously not amenable to analysis.

A universal analyser based on this principle would be applied not only for blood analysis but for investigation of different biological microstructures.

Unfortunately, despite the great potentialities and advantages of cytometric blood analysis by the electronic methods the latter have still not been sufficiently adopted in laboratory practice. In the light of the resolution of the Central Committee of the Communist Party of the Soviet Union and the Council of Ministers of the U.S.S.R. "On Measures for the Further Improvement of Medical Services and Protection of the Health of the Population of the U.S.S.R." it is necessary to work out and arrange for the mass production of industrial models of automatic blood analysers.

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VOL. 5 196

# STUDY BY THE METHOD OF AUTORADIOGRAPHY OF THE INCORPORATION OF RADIOACTIVE SULPHUR-LABELLED CYSTEAMINE, METHIONINE AND SODIUM SULPHATE*

L. N. ZHINKIN and A. A. ZAVARZIN

Institute of Experimental Medicine, U.S.S.R. Academy of Medical Sciences, Leningrad

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A NUMBER of investigators have now shown that the effect of X-rays may be somewhat inhibited. Cysteamine $\beta$-mercaptoethylamine) has a preliminary protective action as shown by Bacq [1]. The value of this preparation has been confirmed in a number of radiation studies [2–4].

Since the mechanism of action of $\beta$-mercaptoethylamine and other protective compounds still largely remains unclear, it was of interest to study the course of its distribution in the body.

For this purpose we used the method of autoradiography to reveal the link between the labelled compound and definite morphological structures. However, in the case of MEA it is not easy to establish the fate of this compound merely from the incorporation of radioactive sulphur. In fact, not being an amino acid, MEA cannot enter into the synthesis of the polypeptide skeleton of the protein molecule. Incorporation of radioactive sulphur into the structure of the molecule in this case may indicate not incorporation of MEA but incorporation of radioactive sulphur previously split off from the MEA and with therefore, a different fate from that of the MEA itself.

These considerations led us to study besides MEA, the incorporation into the same tissues of sulphur-labelled methionine and sodium sulphate. It is known that $^{35}$S-methionine is incorporated chiefly into the proteins [3–5]. The inorganic sulphate accumulates in the main in the sulphated mucopolysaccharides [6–9].

Comparison of the course of the distribution of radioactive sulphur introduced in the form of three different compounds was aimed at revealing the specific aspects of the incorporation of $^{35}$S-MEA into different tissues.

## MATERIAL AND METHODS

The experiments were carried out on adult white rats injected subcutaneously with sulphur-labelled MEA, methionine or sulphate in a dose of 0·5 $\mu$c per g. Since MEA is rapidly destroyed on contact with air, the solution was injected into the animals immediately after opening the ampoule. The ex-

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perimental animals were killed by decapitation over a period from 15 min to 2 days after injection of the tracer. Most of the material was fixed with Carnoy's fluid, in supplementary experiments with formalin or alcohol. The material was embedded in paraffin; after removal of paraffin a sub-layer of gelatine was applied to the sections and then a photographic emulsion, type R(NIKFI).

The experiment was run in three variants: (1) study of the pattern of incorporation of $^{35}$S-MEA; (2) comparison of the incorporation of MEA and methionine; and (3) comparison of incorporation of MEA and sodium sulphate.

The emulsion was applied at the same time to the sections of the different organs obtained from the animals of each series of experiments and killed at different times; this made it possible to obtain comparable results without introducing a correction for the decay of the isotope. The autoradiographs were stained with haemalum-eosin or azure-eosin. The quantitative indications of the intensity of incorporation were found by track counting using the grid of an ocular micrometer of 100 $\mu^2$ area. The average was deduced from a count of 50, 100 or 200 squares.

To bring out more clearly the differences in the incorporation of the radioactive sulphur of MEA, methionine and sodium sulphate in the organs investigated, we studied, as controls, the incorporation of radioactive sulphur into the liver and the blood preserved in sections within the lumen of the vessels. Besides the autoradiographs in which a count was made of the number of tracks, in a number of cases with prolonged exposures, it was possible to obtain "contrast autoradiographs" which gave the general picture of incorporation and distribution of radioactive sulphur.

## RESULTS

In the first experiments, a pilot series, the course of incorporation of $^{35}$S-MEA into the tissues of the liver, tongue, small intestines and bone marrow was investigated.

After 22 days' exposure we obtained only "trace autoradiographs" of the different organs of the animals and the intensity of incorporation was estimated by counting tracks. Contrast autoradiographs could not be obtained in this series. The results of the estimate are shown in Table 1.

TABLE 1

| Tissues | Time after injecting $^{35}$S (hr) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 6 | 12 | 24 | 48 | 120 |
| | Number of tracks from $^{35}$S per 100 $\mu^2$ | | | | | | |
| Liver | 0·35 | 0·5 | 0·85 | 0·5 | 0·5 | 0·35 | 0·25 |
| Tongue | | | | | | | |
| Connective tissue | 1·45 | 1·45 | 1·44 | 1·45 | 0·58 | 0·35 | 0·36 |
| Muscle fibres | 2·1 | 1·45 | 0·94 | 0·69 | 0·39 | 0·28 | 0·22 |
| Arteries of muscle type | | | | | | | |
| tunica intima | 4·47 | | 4·2 | 3·45 | 2·82 | 1·22 | 0·87 |
| tunica media | 1·32 | — | 1·75 | 0·9 | 0·35 | 0·47 | 0·2 |
| Bone marrow | 0·77 | — | — | 1·04 | 0·4 | — | 0·47 |
| Small intestine | | | | | | | |
| Epithelium of crypts | 0·12 | 1·2 | 0·87 | 0·72 | 0·61 | 0·42 | 0·45 |
| Epithelium of apices of villi | 0·52 | 0·97 | 0·73 | 0·78 | 0·66 | 0·57 | 0·24 |

As the results show, the general character of distribution of $^{35}$S-MEA in the tissues investigated was quite similar to that of $^{35}$S-sodium sulphate.

This is indicated by the weak incorporation of $^{35}$S-MEA into the liver and the relatively high content in connective tissue. A selective accumulation of $^{35}$S-MEA was also detected in the goblet cells and various mucus glands.

In addition, peculiarities in the distribution of $^{35}$S-MEA were also demonstrated. They were manifest

in a marked selective accumulation of the radioactive sulphur of MEA in the tunica intima of the lingual artery. Characteristic of the sulphur of MEA was also the intensive accumulation by the striated muscle fibres in the first hours after administration.

For a more detailed analysis of these features of the distribution of radioactive sulphur of MEA, a second series of experiments was carried out aimed at comparing the incorporation and distribution of the radioactive sulphur of MEA and methionine in the walls of vessels with different structure and functional behaviour. In this series, the incorporation of $^{35}$S-methionine and MEA into the wall of the lingual and arciform renal arteries and aorta was investigated. For comparison a study was made in the same animals of incorporation of $^{35}$S into the germ layer of the epidermis, striated muscles, liver and blood.

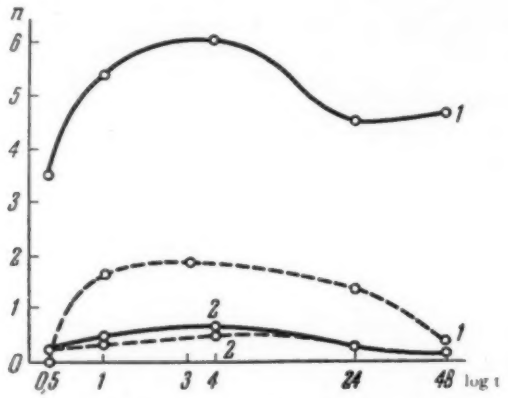


FIG. 1. Incorporation of radioactive sulphur of methionine and cysteinamine into the blood and liver cells; 1, $^{35}$S-methionine; 2, $^{35}$S-cysteinamine. Unbroken line, incorporation of $^{35}$S into liver cells; broken line, incorporation of $^{35}$S into blood. Abscissa, log time in hr; ordinates, number of tracks (n) per 100 $\mu^2$.

Fig. 1 graphically represents the course of incorporation of $^{35}$S of methionine and MEA into the blood and liver cells. The maximum incorporation of the sulphur of methionine was detected in the liver cells after 3 hr, in the blood within only 1 hr. At 24 hr the amount of $^{35}$S diminished slightly but to a greater extent in the blood than in the liver. However, a striking aspect is the marked difference between both curves since uptake by the liver of $^{35}$S at all stages was many times greater than by the blood.

Very little radioactive sulphur of MEA was detected in the blood and liver and the rate of incorporation at all times was quite similar.

Thus, merely from these results it follows that the character of incorporation of $^{35}$S-MEA appreciably differed from that of the radioactive sulphur of methionine. No less striking are the differences on comparing the rate of incorporation of $^{35}$S-MEA and methionine into the wall of the lingual artery, the striated muscles and the germ layer of the epidermis (Fig. 2). It is clear that the inner membrane of the lingual artery (tunica intima) incorporated an equal amount of $^{35}$S-MEA and methionine; the tunica media and the striated muscles incorporated more methionine than MEA and finally, the germ layer of the epidermis contained tens of times more sulphur of methionine than of MEA. If we take into account that the sulphur of MEA has a much lower content in the blood than that of methionine, then the differences detected in the distribution of the sulphur of MEA and methionine are even more arresting. This is particularly well illustrated by the rate of accumulation of radioactive sulphur in the inner membrane of the artery of the muscular type, in this case the amount of radioactive sulphur of methionine was the same as in the blood. Three times as much radioactive sulphur of MEA was incorporated into the tunica intima of the artery than in the blood.

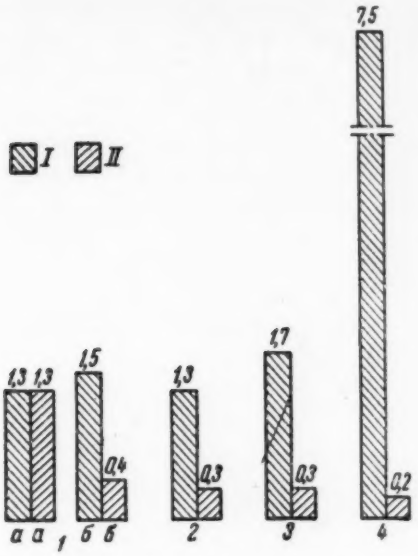


FIG. 2. Relation between incorporation of radioactive sulphur of cysteinamine and methionine: 1, $^{35}S$ in lingual artery; 2, in striated muscle; 3, in blood; 4, in germ layer of lingual epithelium. 3 hr after injection. I, methionine; II, cysteinamine; a, tunica intima; b, tunica media.

Particularly marked differences were detected in the wall of the aorta in which brown fatty cellular tissue was always seen in the sections. An hour after injection of $^{35}S$-MEA intense blackening of the emulsion was observed over the wall of the vessel (Fig. 3(a)). The fatty cellular tissue incorporated a negligible amount of radioactive sulphur and the emulsion here remained clear (Fig. 3(b)). On injection of methionine, on the other hand, the wall of the aorta took up comparatively little isotope, whereas in the fatty cellular tissue the amount of $^{35}S$ was considerable.

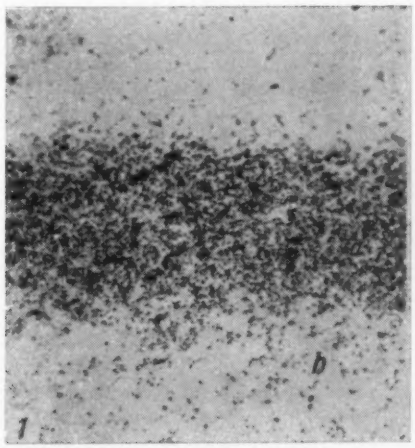


FIG. 3. Contrast autoradiograph of wall of aorta 1 hr after injection of cysteinamine; a, wall of aorta; b, fatty cellular tissue. Exposure 25 days; magnification ×250.

Comparison of the amount of radioactive sulphur in the wall of the aorta with that in the blood shows that in the case of MEA a marked selectivity of accumulation was observed. On injection of methionine its amount was smaller than in the blood. The results indicate that different substances act as substrates for incorporation of $^{35}$S-MEA and methionine.

Study of the course of incorporation and distribution of $^{35}$MEA in the wall of the aorta, the lingual artery and the arciform artery of the kidney showed that this process displays a common pattern for all the vessels investigated (Fig. 4). During the 4 hr after injection of MEA the rate of incorporation of $^{35}$S rose rapidly, after a day the amount fell by half and persisted at this level in the subsequent days.

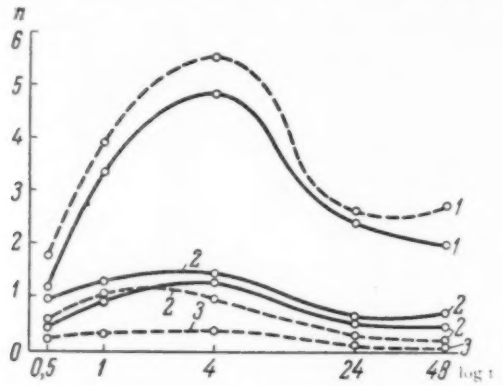


FIG. 4. Incorporation of $^{35}$S-cysteinamine into various vessels: 1, aorta; 2, renal artery — unbroken line, tunica intima, broken line, tunica media; 3, lingual artery. Abscissa, log time in hr; ordinates, number of tracks.

However, each vessel studied had its own peculiarities. The maximum rate of incorporation at all stages was detected in the wall of the aorta, the tunica media of the aorta incorporating somewhat more $^{35}$S than the tunica intima. In the artery of the tongue, on the other hand, a considerably higher uptake of $^{35}$S in the tunica intima was observed. Finally, the arciform artery of the kidney occupies in this respect an intermediate position. Analysis of these peculiarities suggests that the intensity of the selective accumulation of radioactive sulphur of MEA is related to the degree of development of the elastic framework of the wall of the vessel.

In order to confirm this pattern, in the same rat 4 hr after injection of MEA, different zones of the aorta, the pulmonary vein, the endocardium, the hyaline and elastic cartilages and the tunica propria ductus defferens were studied. The most intensive uptake was detected in the abdominal part of the aorta, somewhat less in the pulmonary vein, rich in elastic fibres. The endocardium and the hyaline cartilages gave uniform incorporation — three times greater than for the liver. Incorporation was somewhat less in the elastic cartilage and the tunica propria ductus defferens. Since the elastic cartilage and the ductus defferens, with a rich network of elastic fibres, did not give the same rate of incorporation as the vessels it may be considered that the selective accumulation of the sulphur of MEA is largely bound up with structures specific for the vascular wall.

Comparison of the microphotographs in Fig. 5 shows that the rate of incorporation of radio active sulphur and the pattern of its distribution in the vascular wall precisely corresponds to the degree of development and the pattern of distribution of the elastic structures. The region of maximum incorporation of $^{35}$S in the arteries of the muscular type was localized in the tunica intima — in the region of the membrana elastica interna (Fig. 5(a) (c)). In the vesesls of the elastic type with uniform distribution of the elastic membranes in the inner and median membranes (Fig. 5 (b)) intense blackening of the emulsion was observed both over the inner and the median membrane (Fig. 5 (d)).

All the findings on incorporation of the sulphur of MEA compel one to suppose that it occurs

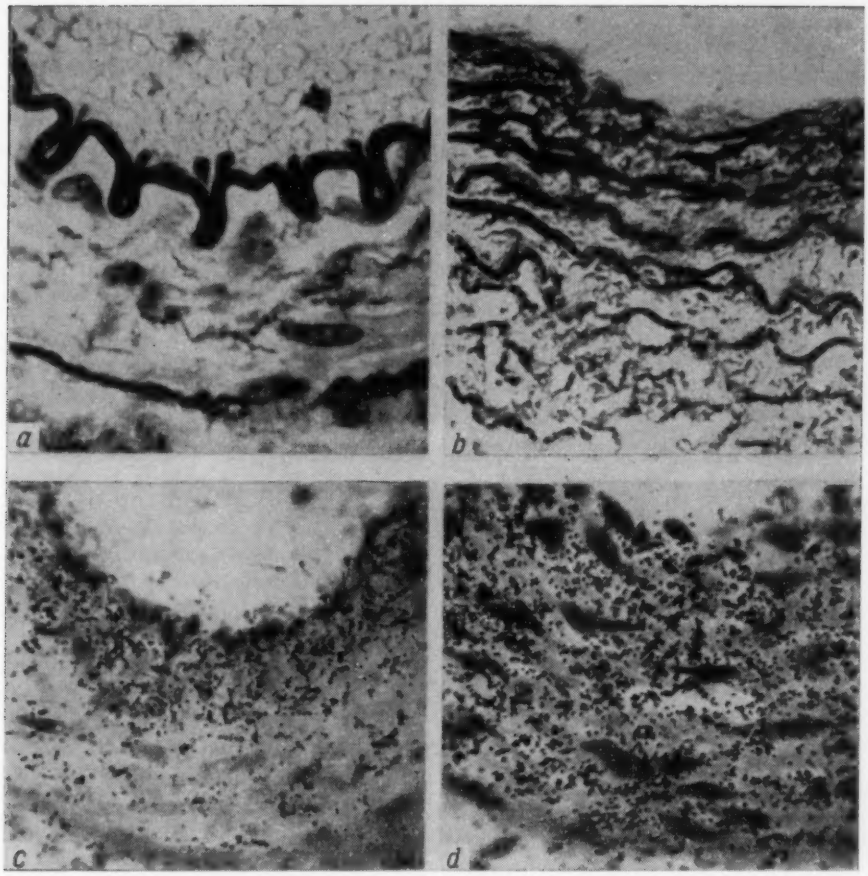


FIG. 5. Tranverse sections of wall of artery of radix linguae (a, c) and pulmonary vein (b, d): a and b, staining with orcein; magnification ×360; c and d, autoradiographs 4 hr after injection of $^{35}$S-cysteinamine, exposure 20 days, staining with haematoxylin–eosin.

in a similar manner to sodium sulphate ($Na_2{}^{35}SO_4$). This is suggested by the incorporation into the cartilage and connective tissue and also by the findings of Voronin who demonstrated intensive incorporation of $^{35}$S-MEA into the mucus glands—similar to incorporation of radioactive sulphur observed after injection of sodium sulphate. Accordingly, the next series of experiments was aimed at comparing the rate of incorporation of the radioactive sulphur of MEA and sodium sulphate.

The comparison was made on animals killed 4 hr after injection of the tracers.

Incorporation of radioactive sulphur was studied in the wall of the aorta, the lingual artery, the pulmonary vein, the endocardium and valves of the heart, cartilage and sclera. In the aorta considerably greater accumulation of radioactive sulphur of MEA (about twice as much) was observed than for sodium sulphate.

The results of the track counts in other organs are shown in Fig. 6. The pulmonary vein and the tunica intima of the lingual artery also incorporated considerably more sulphur from the MEA. As for the cartilage, sclera and the valves of the heart, in all cases the sulphate sulphur was incorporated more intensively than that of MEA.

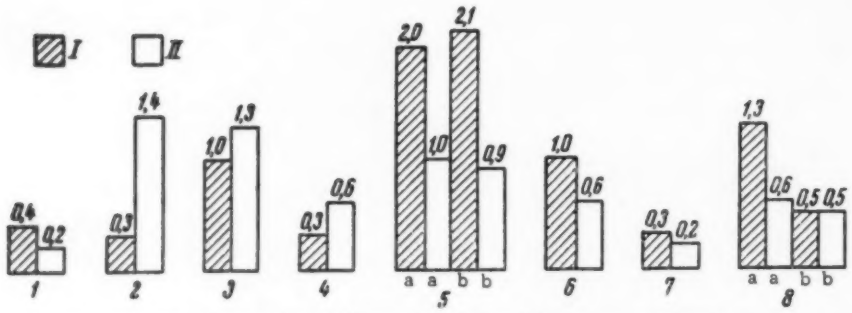


FIG. 6. Relation between incorporation of $^{35}S$-cysteinamine and $^{35}S$-sodium sulphate into various organs 4 hr after injection; I, mercamine; II, sulphate. 1, blood; 2, valves of heart; 3, cartilage; 4, sclera; 5, pulmonary vein; 6, endocardium; 7, lingual muscle; 8, lingual artery; (a) tunica intima, (b) tunica media.

Thus, despite the similar character of distribution of the sulphate sulphur and that of MEA into the vascular wall definite differences were found. They were detected either in the inner and median membranes (aorta and pulmonary vein) or only in the inner membrane (artery of muscular type).

The raised specific accumulation of the sulphur of MEA in the walls of the blood vessels shows that the substrate for the incorporation of radioactive sulphur of MEA was somewhat different from that for the radioactive sulphur of sodium sulphate.

For a more detailed identification of the substrate in the aorta sections, an experiment was carried out with trichloracetic acid. The sections of the aorta of the rat which 4 hr before fixation of the material was injected with MEA were divided into four groups: (1) sections before application of the emulsion were subjected for 15 min to treatment with 5% trichloracetic acid at 90° (following Schneider); (2) sections were treated with water at 90°; (3) sections were treated with cold water; and (4) sections were not treated at all.

The emulsion was applied simultaneously to all the sections. After 10 days' exposure sections were developed. The results of the track counts are shown in Table 2.

TABLE 2

| Tissue | Number of tracks of $^{35}S$ per 100 $\mu^2$ | | |
|---|---|---|---|
| | Controls | Trichloracetic acid at 90° | Water at 90° |
| Tunica intima | 2·08 | 1·03 | 1·02 |
| Tunica media | 2·23 | 1·4 | 1·28 |
| Tunica adventitia | 1·0 | 0·7 | 0·74 |
| Fatty cellular tissue | 0·24 | 0·28 | 0·24 |

After treatment of the sections incorporation into the tunica intima and the tunica media diminished by almost half for both the action of trichloracetic acid and hot water. Less $^{35}S$ was removed from the tunica adventitia and no difference was observed between the controls and the treated sections for the fatty cellular tissues. Since hot water and trichloracetic acid washed out the radioactive sulphur from the sections in an equal amount, then in this case the effect was due to treatment with hot water. Washing in cold water did not give this effect.

In the next experiment, repeated twice, incorporation of $^{35}S$ of MEA and sodium sulphate was compared after using different fixatives. Neighbouring areas of the aorta 4 hr after introduction of

VOL. 5 1960

the tracers were fixed with (1) Carnoy's fluid; and (2) alcohol and formalin. The sections fixed with the various fixatives were sealed to the same slide which made possible the most accurate comparison between them.

A track count (Fig. 7) after injection of the sulphate showed no differences in the rate of incorporation of $^{35}S$ into the aorta wall. The amount of radioactive sulphur in the sections with both fixatives

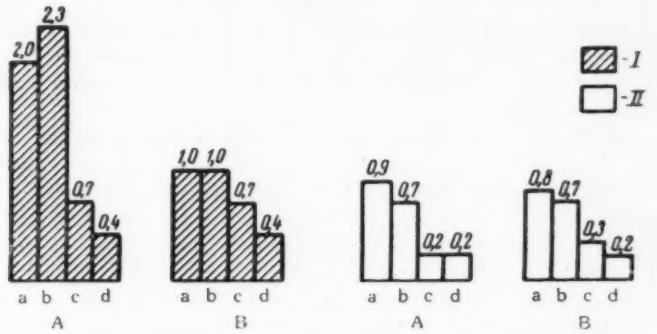


FIG. 7. Incorporation of $^{35}S$-cysteinamine and sulphate into the wall of the aorta 4 hr after injection and fixation by Carnoy's fluid on alcohol-formalin; I, mercamine; II, sulphate. A, fixation with Carnoy's fluid; B, fixation with alcohol and formalin. a, tunica intima; b, tunica media; c, tunica adventitia; d, blood plasma.

was virtually the same. On injection of MEA after fixation with formalin the number of tracks in the tunica intima and the tunica media was half that after fixation with Carnoy's fluid. The collagen bundles and the fatty cellular tissue gave identical uptake of $^{35}S$-MEA for both fixatives.

## DISCUSSION

In comparing the results obtained we must first of all emphasize the sharp difference in the distribution of $^{35}S$ of MEA and methionine. Despite the presence in both compounds of SH groups, their substrates for incorporation were different. Since it has now been firmly established that the sulphur of methionine detected in the autoradiographs is bound with sulphur-containing proteins [5, 10, 11, 12], it may be considered that on injection of animals with MEA, it is not joined to the protein molecules. In any case, it was not possible in our experiments to obtain any distinct evidence pointing to the participation of the radioactive sulphur of MEA in the synthesis of the proteins.

In contrast to the marked differences in the character of distribution of radioactive sulphur of methionine and MEA, the course of accumulation and the nature of the distribution of $^{35}S$-MEA and sodium sulphate was largely similar. Since the bulk of the sodium sulphate, as has been shown by a number of investigators [7–10], participates in the synthesis of mucopolysaccharides, the agreement obtained between the course of distribution of the radioactive sulphur of MEA and sodium sulphate was not fortuitous. It indicates that on injection of MEA into the body, oxidation occurs of a certain part to sodium sulphate, which accounts for the agreement of the results obtained by the method of autoradiography.

However, in the vascular system, namely in regions where the elastic fibres are most developed, besides the sulphur of the sulphated mucopolysaccharides, sulphur forming part of another chemical compound was detected. This compound apparently possesses a free amino group, since on fixation with formalin it binds itself with formaldehyde and is removed from the tissue. Since radioactive sulphur is thereby removed, it may be assumed that this substance is MEA or a related compound.

The binding of this compound in the vascular wall is of a different character to the incorporation of the sulphur of sodium sulphate and methionine. The radioactive sulphur of methionine and sodium sulphate on participating in the synthesis of proteins or mucopolysaccharides is inseparably bound with the structures and is not removed by various fixatives or heat. The sulphur of MEA or its de-

rivatives is labilely bound in the vessels and on interaction with formaldehyde or on treatment with hot water is readily washed out from the preparation.

In our view, the results obtained are not only of special interest but are of a more general importance in elucidating the distinctive features of metabolic processes in the vascular walls.

## SUMMARY

(1) Comparison of the distribution and course of incorporation of the radioactive sulphur of cysteinamine, sodium sulphate and methionine in different tissues of white rats has shown that incorporation of $^{35}$S-cysteinamine occurs along similar lines to sodium sulphate and sharply differs from incorporation of $^{35}$S-methionine.

(2) Specific for the $^{35}$S of cysteinamine is the sharply marked selectivity of its accumulation in the vascular walls. The rate of incorporation and localization of the region of maximum incorporation of $^{35}$S of cysteinamine in the walls of the vessels is directly related to the degree of development of the elastic structures.

(3) About half the $^{35}$S-cysteinamine detected in the autographs of the aorta wall is incorporated in the molecules of the sulphated mucopolysaccharides. A considerable part of the $^{35}$S of cysteinamine incorporated into the vascular walls forms part of a compound similar in its chemical nature to mercamine. This radioactive sulphur is labilely bound with the histological structures and is removed from the sections on heating and on fixation with formalin.

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VOL. 5 196

# FUNCTION OF RECOVERY ON IRRADIATION OF ORGANISMS

K. K. AGLINTSEV

Khlopin Radium Institute, U.S.S.R. Academy of Sciences, Leningrad

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RADIATION injuries to the body in a number of cases have a reversible character. In these cases it is usually said that a process of recovery occurs. The relation between the radiation dose and the effect produced by it in the presence of recovery will depend on the dose rate. Probably it is futile to attempt to describe these processes by means of simple mathematical equations, particularly since the equations cannot disclose the nature of the process of recovery. An attempt is made below to establish certain properties of the dependence of the effect of irradiation on the dose rate. The arguments presented must be considered as a formulation of the problem of recovery and not its solution.

We introduce the following notation: $D$, radiation dose, $P$, instantaneous value of the dose rate during a very short interval of time $dt$ from the moment $t$ to the moment $t+dt$. The dose for this interval of time $dt$, will be equal to $Pdt$. The full value of the dose $D$ for a certain finite period of time from moment 0 to moment $T$ will be equal to the sum of the values of $Pdt$ for all the very short intervals of time $dt$, i.e. the dose is determined by the relation

$$D = \int_0^T P dt. \tag{1}$$

If the dose rate retains a certain constant value $P_0$ then in the graph (Fig. 1) the dependence of $P$ on $t$ is represented by a horizontal straight line located at a distance $P_0$ from the abscissa. The dose $P_0 dt$ will be equal to the area of the shaded rectangle with height $P_0$ and base $dt$. The dose $D$ for time $T$ obviously will be equal to the sum of the areas of all the small rectangles. The area of the shaded figure (the rectangle with height $P_0$ and base $T$) $= P_0 T$, i.e. the dose $D$.

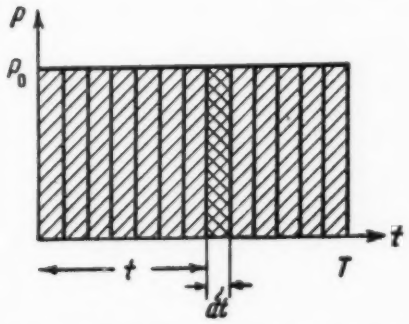


FIG. 1. Calculation of dose for constant dose rate.

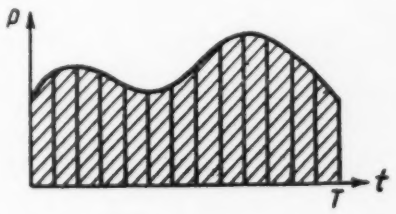


FIG. 2. Calculation of dose for variable dose rate.

* Biofizika **5**: No. 6, 740–744, 1960.

In the case in which the dose rate $P$ varies in time the dependence of $P$ on $t$ must be represented graphically. Such a construction leads to the same relation (1). However, in the case of a variable dose rate, the height and areas of the small rectangles $Pdt$, making up the area of the shaded figure, will differ as Fig. 2 shows.

We now turn to consideration of the rôle of the process of recovery. We shall confine ourselves to the case of irradiation of the body with a constant dose rate $P_0$. We shall introduce the purely formal notion of the "radiation memory" of the irradiated body and consider that accumulation of the biological action of radiation is expressed in the fact that the organism "remembers" the fact that it has already received a certain dose $P_0dt$. We shall denote the radiation memory of irradiation for the time from $t$ to $t+dt$ by $p_0dt$, i.e. recall of the fact that the organism has already received a certain dose $p_0dt$.

We shall consider that the biological dose for a certain interval of time will be proportional to the cumulative sum of the values of radiation memory for this period of time. The radiation effect for one of the preceding intervals of time $dt$ is proportional to $pdt$ and the effect of irradiation for the whole time 0 to $T$ is proportional to the sum $\int_0^T pdt$. We shall take the constant of proportionality as equal to 1. Hence, the biological dose $D_b$ is defined by the relation:

$$D_b = \int_0^T pdt.$$

We shall interpret the absence of recovery as the retention by memory of the value $pdt$ as an unchanged non-diminishing value $p_0dt$. Graphically, this may be represented by the same Fig. 1, where instead of a constant dose rate $P_0$ we must substitute a constant value of radiation memory $p_0$.

The process of recovery leads to a gradual smoothing out of the radiation memory from certain. initial values $p_0$ to values successively diminishing with time. Hence, the rectangles in Fig. 1 will gradually decrease and instead of Fig. 1 we obtain for successive moments of time the picture depicted in Fig. 3. Here, the values of $p_0$ are depicted for the moments of time $\Delta t$, $2\Delta t$, $3\Delta t$, ... $10\Delta t$ and each rectangle for time $\Delta t$ falls by 20 per cent and after $11\Delta t$ to zero. The initial value $p_0$ is taken as equal to 25. With passage of time the radiation memory depicted in Fig. 3 falls successively from the initial value 25 o the values 20, 16, 13, 10, 8, 6, 4, 2, 0. The areas of the figures for the moments of time $\Delta t$, $2\Delta t$, $3\Delta t$,... are respectively equal to: 25, 45, 61... Fig. 3 indicates the value of the area for each figure and Fig. 4 shows the rise in the sum $\int_0^T \bar{p}dt$ for the case $T = 12\Delta t = 12$. It is taken that $\Delta t = 1$.

If the value $p(t)$ during a certain interval of time $\tau$ becomes zero, then we shall speak of complete recovery and the value $\tau$ we shall term the "time of complete recovery". Fig. 3 corresponds to the duration of complete recovery $\tau = 12$. Obviously, irradiation at a given moment $t$ will no longer have any effect after moment $t+\tau$ and therefore, the biological dose $D_0$ will not increase after time $\tau$ as shown in Fig. 4.

The law of the diminution in time of the values $p(t)$ is unknown. Possibly it can be established from experimental data. We can now only put forward certain hypotheses on the form of the function $p(t)$. It must first of all be assumed that the derivative of the function $p(t)$ is negative or equal to zero since this function diminishes or maintains a constant value. Consequently,

$$\frac{dp}{dt} = -f(t). \tag{3}$$

where $f(t)$ is unknown but essentially a positive function or in the absence of recovery zero.

The simplest course is to assume that $f(t)$ is a power function, i.e.

$$\frac{dp}{dt} = -knt^{n-1} \tag{4}$$

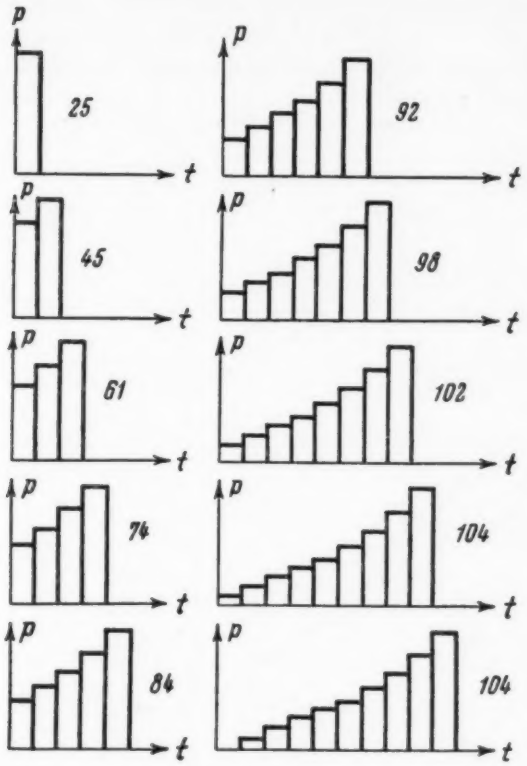


FIG. 3. Biologically significant dose with variable duration of irradiation in presence of recovery (initial value $P_0$ taken as 25).

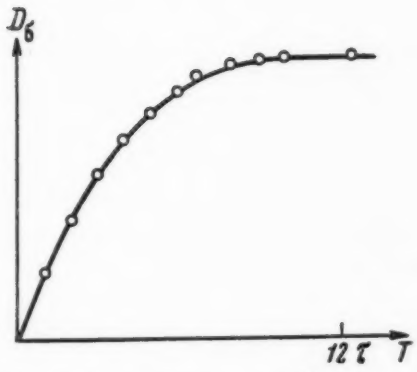


FIG. 4. Variation of biologically significant dose with time of irradiation in presence of recovery.

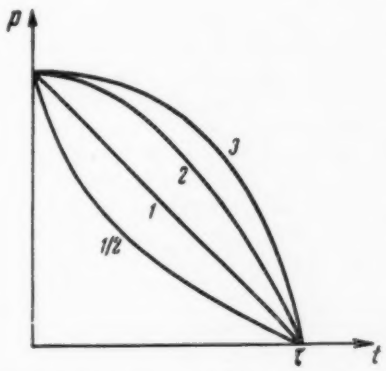


FIG. 5. Various forms of "radiation memory" as a function of time with equal values for time of complete recovery.

and consequently:

$$p = p_0 - kt^n. \tag{5}$$

We shall make the simplifying assumption that the function $f(t)$ does not change its form with time, i.e. the values $k$ and $n$ are constant.

The duration of the complete recovery $\tau$ is defined from the condition

$$\tau = \sqrt[n]{\frac{p_0}{k}}, \tag{6}$$

and the coefficient $k$ is equal to

$$k = \frac{p_0}{\tau^n}. \tag{7}$$

Fig. 5 gives the curves for the function $p(t)$ for the values $n = 1/2, 1, 2, 3$; identical values of time $\tau$ were selected. Fig. 6 gives the same functions but here the intervals of time $\tau_{1/2}$ during which the value of the function $p(t)$ falls to a half are the same.

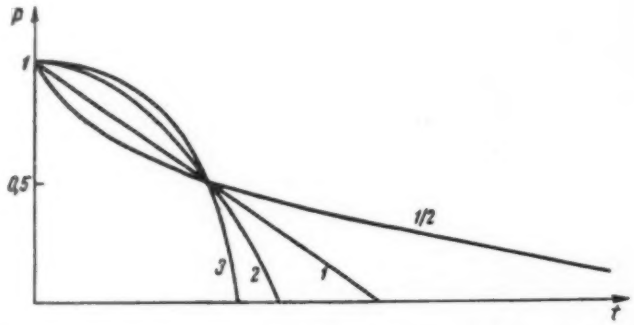


FIG. 6. Various forms of "radiation memory" as a function of time with equal values of time of half-recovery.

The straight line corresponds to the value $n = 1$. In this case the radiation memory falls off uniformly in time. For the value $n = 2$ or 3 the rate of decline in the radiation memory constantly increases and for $n = 2$ is proportional to the interval of time after termination of irradiation. The rôle of the process of recovery here becomes increasingly noticeable. For the value $n = 1/2$ the radiation memory declines at first rapidly and then more slowly.

We shall find the value $D_b$ from the relation (2) taking into consideration the hypotheses made with respect to the form of the function $p(t)$. Two cases have to be distinguished: $T < \tau$ and $T > \tau$.

The case $T < \tau$ may occur either when the duration of radiation is less than the time of complete recovery or when complete recovery does not occur at all. In the latter case $\tau$ is an imaginary value but it can readily be seen that this does not effect the calculations given below. It is simply necessary to assume that the value $\tau$ is very large and recovery cannot be attained.

Integration of (2) gives

$$D_b = \int_0^\tau p\,dt = p_0 T - \frac{kT^{n+1}}{n+1}, \tag{8}$$

or inserting the value $\tau$

$$D_b = p_0 T\left[1 - \frac{1}{n+1}\left(\frac{T}{\tau}\right)^n\right]. \tag{9}$$

Case $T > \tau$ corresponds to the case in which complete recovery occurs and the duration of the radiation is greater than the time of recovery. In this case the limit of integration must be $\tau$ since ir-

radiation for the time exceeding the $\tau$ will disappear from the radiation memory or in other words, the process of recovery destroys the after-effects of irradiation of the body. Integration gives

$$D_b = \int_0^T p\,dt = p_0\tau - \frac{k\tau^{n+1}}{n+1}, \tag{10}$$

or

$$D_b = p_0\tau\,\frac{n}{n+1}. \tag{11}$$

In the absence of recovery we would have:

$$D_0 = p_0 T. \tag{12}$$

For the ratios $D_b/D_0$ on the basis of (8)–(12) we find

$$\text{(a)}\quad T < \tau\quad \frac{D_b}{D_0} = \left[1 - \left(\frac{1}{n+1}\right)\left(\frac{T}{\tau}\right)^n\right], \tag{13}$$

$$\text{(b)}\quad T > \tau\quad \frac{D_b}{D_0} = \frac{n}{n+1}\left(\frac{T}{\tau}\right). \tag{14}$$

As can be seen from relation (13) and (14) the sum of the values of the radiation memory is always less than the value of the radiation dose. Fig. 7 depicts the dependence of the ratio $D_b/D_0$ on the duration of radiation for $n = 1/2, 1, 2, 3$ for equal values of $\tau$. As Fig. 7 shows, for $T = 0$, $D_b/D_0 = 1$, i.e. for brief irradiation the factor of recovery does not have time to make itself felt. As the duration of irradiation increases, the ratio $D_b/D_0$ falls off, corresponding to an increase in the rôle of recovery. The curves for different values of $n$ differ little from each other. A certain difference is observed in the initial portions of the curves and in the values $D_b/D_0$ for $T > \tau$.

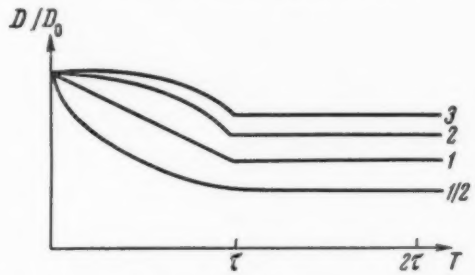


FIG. 7. Relation between biologically-significant dose and radiation dose with variable duration of radiation and a given value ($\tau$) of time of complete recovery.

The reciprocal $D_0/D_b$ shows how many times it is necessary in the presence of the process of recovery to increase the dose rate to obtain the same effect as obtained by dose $D_0$ in the absence of recovery.

We shall now compare continuous irradiation and repeated irradiation with fractionated doses. We shall assume that in the latter case irradiation is delivered by practically instantaneous pulses separated in time from each other by the intervals $T_0$. We shall denote by $\delta$ the dose from one pulse and by $D_b$ the dose for the series of $N$ pulses. In the absence of recovery the series of $N$ pulses will give a dose $D_0 = N\delta$. In the presence of recovery radiation memory of each pulse will obviously be smoothed out and the dose $D_b$ will be less than the dose $D_0$. We shall consider the relation between doses $D_b$ and $D_0$ for different relations between $t$ and $T_0$. Obviously the absence of recovery may be interpreted as $T/\tau = 0$. If the duration of recovery is less than the pause $T_0$ then the effect of each pulse will be compensated by recovery and here the dose $D_b = \delta$. Hence, we can confine ourselves to consideration of cases in which the ratio $T/\tau$ lies within the limits from 0 to 1, i.e. $0 \leqslant T/\tau \leqslant 1$.

Fig. 8 depicts the case $T/\tau = 0{\cdot}5$, i.e. where the duration of complete recovery is equal to double the duration of the pause $T_0$. The vertical lines show individual pulses of irradiation, the sloping lines the fall-off in the radiation memory after each pulse. We shall confine ourselves here to case $n = 1$, i.e. uniform diminution in the radiation memory. The value of the radiation memory at a certain moment

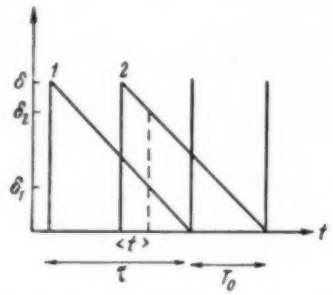


FIG. 8. Biologically significant dose for pulsed irradiation ($\tau = 2\,T$).

$t$ after the pulse is obviously equal to the sum $\delta_1+\delta_2$ where $\delta_1$ and $\delta_2$ are the values of the dose for each of the pulses. Inspection of the triangles formed by the abscissa, the vertical and the sloping lines convincingly show the validity of the following relations:

$$\delta_1 = \frac{T_0 - t}{2\,T_0}\,\delta; \quad \delta_2 = \frac{2\,T_0 - t}{2\,T_0}\,\delta; \quad \delta_1 + \delta_2 = \frac{3\,T_0 - 2t}{2\,T_0}\,\delta. \tag{15}$$

The sum $\delta_1+\delta_2$ varies from the value $0{\cdot}5\,\delta$ to the value $1{\cdot}5\,\delta$. The first value is obtained directly before the pulse; at that moment the radiation memory from the first pulse has already fallen to 0 and the memory from the second pulse to the value $\delta/2$. The value $1{\cdot}5\,\delta$ is obtained immediately after the pulse; at that moment $\delta/2$ from the first pulse is still maintained and to it is added the dose $\delta$ of the second pulse.

An analogous construction enables us to obtain for the case $T_0/\tau = 1/3$ the following relation:

$$\delta_1 + \delta_2 + \delta_3 = \frac{6\,T_0 - 3t}{3\,T_0}\,\delta, \tag{a}$$

and here the sum $\delta_1+\delta_2+\delta_3$ varies between the values $\delta$ and $2\,\delta$. In the general case when $T/\tau = 1/k$, the total dose from $k$ pulses varies from $\dfrac{k-1}{2}\,\delta$ to $\dfrac{k+1}{2}\,\delta$.

If, $\tau > NT_0$ then, obviously it is only necessary to calculate the total dose from all the $k$ pulses. As Fig. 9 shows, the dose from the $k^{\text{th}}$ pulse is defined from the relation:

$$\frac{\delta_k}{\delta} = \frac{\tau - (N-k)\,T_0}{\tau}, \tag{b}$$

the total dose from all $N$ pulses equals:

$$D_b = N\delta - \frac{T_0}{\tau}\,\frac{N(N-1)}{2}\,\delta. \tag{c}$$

If, in the latter relation we posit $T_0/\tau = 0$, then we obtain the obvious result corresponding to the absence of recovery:

$$D_b = N\delta.$$

Fig. 10 depicts the dependence of the dose $D_b$ on the ratio $T/\tau$. For $T/\tau = 0$ the dose $D_b$ has the value $N\delta$; the same value corresponds to the absence of recovery. For values $T/\tau$ between 0 and

1 the dose varies between the two values — minimum and maximum; one of them is obtained before the pulse, the second immediately after. As the ratio $T/\tau$ increases the minimum and maximum values in conformity with the remarks made above decrease to the values 0 and $\delta$. Impairment of recovery leads to a shift upwards and to the left.

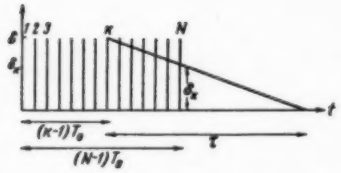


FIG. 9. Biologically significant dose for pulsed irradiation ($\tau = kT$).

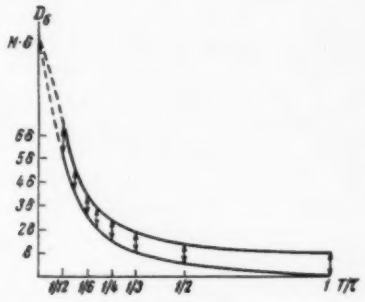


FIG. 10. Biologically significant dose as a function of $T/\tau$.

# LETTERS TO THE EDITORS

## DEGREE OF POLYMERIZATION OF DEOXYRIBONUCLEIC ACID OF ANIMAL ORIGIN*

G. P. GEORGIYEV and V. A. STRUCHKOV

Severtsov Institute of Animal Morphology and the Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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IN the last few years it has been shown that DNA with a molecular weight of $50\text{–}60\times10^6$ can be isolated from phage by means of lysis and centrifugation in a density gradient [1–5]. Measurements of the molecular weight were made autoradiographically. Such "superpolymer" DNA preparations are distinguished by high characteristic viscosity ($[\eta]$), varying according to different workers from 110 to 200 dl/g as against 70 dl/g for the normal DNA preparations of molecular weight $8\times10^6$, and by a higher sedimentation coefficient. On mechanical treatment (agitation, forced passage through a capillary) the polymer breaks down into smaller particles with a moleculer weight of $8\text{–}10\times10^6$. DNA of this maximum molecular weight can be obtained from animal tissues.

All the methods of isolating DNA described in references [6–8] start with animal tissues and include shaking the suspension of tissue or nucleoprotein with chloroform, phenol or detergent. Therefore it was natural to assume that the comparatively low molecular weights obtained for DNA of animal origin may be connected with a breakdown of its particles into smaller units during the mechanical isolation treatment.

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The present paper reports findings showing that if on isolation of DNA harsh mechanical treatment is avoided, it is possible to obtain preparations with much higher viscosity and rate of sedimentation than in normal conditions. Such superpolymer preparations of DNA are extremely unstable on mechanical treatment.

## METHODS

The method for obtaining the DNA is described below.

To determine the amount of DNA in the preparations, which also contained a considerable amount of RNA, we used either the Webb's method (9) or separated the RNA and DNA according to Schmidt and Tannhauser with subsequent spectrophotometric determination of the DNA. The viscosity of the preparations was determined in Ostwald viscometers at temperatures of 25°–95°. We used 0·0005–0·003% DNA solution in 0·14 M NaCl in 0·01 M phosphate buffer at pH 0·7. The temperature was regulated by a Vobser ultrathermostat.

For the semi-quantitative investigation of the sedimentation rate of the DNA, the method of preparative ultracentrifugation in starch was employed [10].

The centrifuge tubes contained one layer of starch (1 ml packed water-insoluble starch with the gaps filled with 0·14 M NaCl in 0·01 M phosphate buffer at pH 7·0), a diaphragm of filter paper perpendicular to the centrifugal force and 1·0 ml of 0·0025% DNA in the same solvent. Centrifugation was carried out in the small rotor of the UTsP-1 preparative ultracentrifuge for 15 min at 35,000 r.p.m. (90,000 g) at $18 \pm 2°$. After stopping the ultracentrifuge, the aqueous layer was removed from the tubes with a Pasteur pipette and the DNA content determined. From the fall in DNA content in the upper layer, it was possible to determine the fraction of DNA which sedimented as a result of ultracentrifugation and thereby obtain an idea of the sedimentation rate of DNA.

## RESULTS

1. *Preparation of DNA samples in the "superpolymer" state.* In order to obtain DNA in the maximum polymer state, it was necessary to exclude the possibility of enzymatic hydrolysis and mechanical damage from shaking. In order to satisfy the first condition, we chose the phenol method of extraction with Ehrlich ascites carcinoma cells as the test object, which enabled us to dispense with homogenization.

The cells were washed and then suspended in 0·14 M NaCl and an equal volume of water-saturated phenol, pH 8·3 (2 ml 5 N KOH per 100 ml phenol), was added [8]. Mixing was at room temperature for 30 min with very careful circular rotations of the flask and careful pouring from one flask to another. The viscous mixture in polyethylene containers was centrifuged for 20 min at 30,000 g in a MSE SS-25 refrigerated centrifuge. The aqueous phase was drawn off with a wide bore pipette and subjected to similar repeat mild deproteinization.

The viscous aqueous solution obtained after centrifugation to remove the phenol was dialysed for 2 days against 0·14 M NaCl in 0·01 M phosphate buffer at pH 7·0, in the cold. The solutions were all saturated with chloroform. After dialysis they were analysed. In addition to the DNA, they contained RNA and a certain amount of polysaccharides, but the properties of the solutions examined in the present work, as will be shown later, depended entirely on the DNA. Therefore, the DNA was not further purified. However, in the final experiments it was found that the superpolymer DNA was readily sedimented at comparatively low accelerations (80,000–100,000 g) when the low-polymer substances remained in solution. This makes simple purification of DNA possible, a factor we now utilise.

2. *Viscosity of DNA preparations.* The characteristic viscosity $[\eta]$ of the high-polymer preparations of DNA with molecular weight of $6 \div 10 \times 10^6$ obtained from animal tissues is usually about 70–80 dl/g. The DNA preparations obtained by us gave $[\eta] = 120 \div 140$ dl/g for different experiments. It should be noted that in the range of concentrations for which the measurements were made, the viscosity was independent of concentration. The fact that in different experiments we obtained preparations with different characteristic viscosities was obviously dependent on the insufficiently standard procedure of mixing so that there was more chance of mechanical damage to the DNA in some experiments than

VOL. 5 196

in others. We cannot therefore consider $[\eta] = 140$ dl/g as the upper limit for the viscosity of the DNA preparations. It is possible that even milder mechanical treatment during mixing would give even more viscous DNA preparations.

In work with different viscometers we obtained the same values for the viscosity of the same DNA preparation. However, with viscometers with a very high gradient of rate of flow (2000 sec) we obtained much higher values of viscosity for the superpolymer DNA preparations (up to 450 dl/g). This phenomenon of structural viscosity in our DNA preparations, which is absent in normal DNA samples, will be described in detail in a subsequent paper.

If solutions of superpolymer DNA are agitated or forced through the capillary of the viscometer, their viscosity falls considerably. By means of different degrees of mechanical treatment, preparations can be obtained with different values of $[\eta]$. Thus, for one of the DNA preparations $[\eta]$ before treatment was 140, after 1 forced passage through the viscometer capillary it fell to 120, after 3 passages to 105, after 7 to 89 and after 12 to 71 dl/g.

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The fall in viscosity due to mechanical treatment is irreversible. On standing for 5 days, no change in $[\eta]$ was noted either in the case of a solution of the superpolymer or the degraded DNA preparations.

Nor was there virtually any change in viscosity between the preparations after precipitating the DNA with 1·5 volumes of alcohol, carefully collecting the precipitate and redissolving it in the original volume of buffered saline. It is necessary to show extreme care in dissolving the DNA and to avoid rough agitation. These results point to the fact that fall in the viscosity on shaking is based on the phenomenon of thixotropy and a probable reduction in the degree of polymerization of the DNA.

To demonstrate that the high viscosity of the solutions was solely dependent on the DNA, we ran experiments with enzymatic treatment of our preparations. The enzymes, in an amount of 0·5 mg per 0·2–0·3 mg DNA, were added directly to the viscometer after which the viscosity was measured at 25° over 2·5 hr. It was found that addition of deoxyribonuclease reduced the viscosity of the solution within a few minutes to that of the pure solvent. Hence, in the dilutions used by us RNA and the other impurities had virtually no effect on the viscosity of the solution which was wholly accounted for by the DNA.

On the other hand, treatment with ribonuclease, trypsin or chymotrypsin caused only a very minor initial fall in the viscosity of the solution (not more than 3–5 per cent on conversion to the reported viscosity) after which no further changes occurred during 2·5 hr. Since all the enzymes investigated are basic proteins, the slight initial drop in viscosity may be attributed to weak complexing between them and the DNA. If after incubation with ribounuclease, chymotrypsin or trypsin, the solution were treated mechanically then the usual fall in $[\eta]$ occurred. These results confirm that the RNA impurity does not affect the viscosity of the preparations. In addition, they make it unlikely, though do not completely exclude the possible participation of protein bridges between the DNA chains in ensuring the high viscosity of the solutions since, according to Butler *et al.* [11], these bridges are readily digested by chymotrypsin.

Finally, it was of interest to investigate the temperature dependence of the viscosity of DNA. As is known, fall in the viscosity of DNA solutions on heating occurs abruptly at the point of denaturation of the molecules. It was found that the preparations of superpolymer DNA behave likewise. On heating to 85° their viscosity did not vary but on further heating to 90° above fell sharply. Hence, in this respect superpolymer DNA has the property common to the usual twin helix of DNA.

In conclusion, it should be noted that we have also obtained preparations with such high characteristic viscosity (120–140) from calf thymus and chick red cells.

3. *Investigation of sedimentation of DNA.* As we did not have at our disposal an analytical ultracentrifuge we used for comparative assessment of the sedimentation rate of the various DNA preparations the method of preparative ultracentrifugation in starch.

The DNA preparations with $[\eta] = 85$, 100 and 120 were obtained from a preparation with $[\eta] = 170$ by differing degrees of agitation. We give the results of centrifugation of 4 DNA preparations (for details, see section "Methods"):

| $[\eta]$ of DNA preparation | 85 dl/g | 100 dl/g | 120 dl/g | 140 dl/g |
|---|---|---|---|---|
| % DNA left in upper aqueous layer | 60 | 40 | 27 | 18 |
| % DNA passing into starch layer | 40 | 60 | 73 | 82 |

It follows from this table that a direct relation exists between the viscosity of the DNA preparation and the percentage of DNA passing into the starch layer, i.e. the sedimentation rate of DNA. This confirms that the high viscosity of our DNA preparations was actually related to the presence in them of much higher polymer particles.

## DISCUSSION

Our findings show that by excluding rough mechanical handling it is possible to obtain from animal tissues DNA preparations of much higher viscosity and sedimentation rate than are usually obtained. This is obviously linked with the large size of the DNA particles. On our results we cannot indicate how large these particles are. Our best preparations gave $[\eta] = 140$. If we consider that $[\eta]$ is directly proportional to M [12] then the value of M for such preparations will be of the order of $15 \times 10^6$. However, as reported, phage DNA preparations with $M = 50 \div 60 \times 10^6$ may have a value for $[\eta]$ of 120, i.e. apparently for DNA with $M > 8 \times 10^6$, other relationships may exist between M and $[\eta]$ which are connected with the influence of the shape of the molecule, degree of hydration, etc. In any case, $[\eta]$ can hardly rise faster than M [13].

The second problem involving the large dimensions of the DNA particles is also still unclear. Does there exist a continuous highly asymmetrical double helix of DNA or does the enlargement of the particle size arise from branching? Is a rôle played here by protein bridges between the DNA molecules, are all the bonds in the superpolymer DNA uniform or, finally, are there mechanically-weak zones along which the ruptures occur on shaking? Answers to these questions may be given by subsequent physicochemical studies of DNA. Among the results given in this paper the information on the temperature curve for $[\eta]$, the structural viscosity of the DNA and the invariability of $[\eta]$ on treatment of DNA preparations with proteases is of interest for an understanding of the structure of the DNA superpolymer.

## SUMMARY

By means of very mild mechanical treatment of suspensions of Ehrlich ascites carcinoma cells with phenol at pH 8·3, DNA preparations were obtained with considerably higher characteristic viscosity and sedimentation rate than are normal for DNA obtained from animal tissues.

Mechanical treatment of the DNA superpolymer preparations causes a fall in the viscosity and sedimentation rate to values typical of DNA with $M = 5 \div 8 \times 10^6$.

Ribonuclease, trypsin and chymotrypsin do not affect the viscosity of the preparations of superpolymer DNA but deoxyribonuclease rapidly reduces it to zero.

The course of the temperature dependence of superpolymer and normal native DNA is identical.

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* There is a translation into English of this work.

# RNA IN THE GANGLION CELLS OF THE FROG RETINA IN THE DARK AND ON ILLUMINATION WITH CONSTANT AND FLICKERING LIGHT*

I. A. UTINA, N. V. NECHAYEVA and V. YA. BRODSKII

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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THE work of many authors has shown a definite quantitative relation between the functional state of the nerve cell and the RNA content of its cytoplasm [1–3].

A distinct relation between the intensity and duration of light stimulation and the cytoplasmatic RNA content has been described for the retinal ganglion cells of amphibia [4, 5]. In this work, the ganglion cells were excited by flickering light since it is known from electrophysiological experiments that the ganglion cells of the retina are excited chiefly by change in the intensity of light. Many ganglion cells are not active on exposure to constant light [6, 7]. It was thus of interest to find out how the RNA values vary in the cytoplasm of the ganglion cells when subject to constant and flickering light. Should the histochemical aspect agree with the results of electrophysiological research, we would be more confident that change in the level of RNA can be taken as a guide to the degree of excitation of the nerve cells.

To clear up this problem, an experiment was conducted as follows. A batch of frogs (*Rana radibunda*) of the same catch were kept in the dark for a day. The experiments were not done on isolated eye preparations, as is usual in electrophysiological investigations, but on whole frogs since the RNA metabolism of isolated preparations is noticeably disturbed [5]. All the animals prior to the experiment received subcutaneously 1–2 ml 0·1% solution of the curare-like preparation, diplacin. The reason for using diplacin is that for the task set (the effect of constant light) any movement of the eye leading to change in illumination of the retina must be absolutely avoided. In the work mentioned above [57] it was shown that the retinas of frogs rendered motionless by diplacin and normal ones show virtually no difference in RNA content. Hence, by working with frogs treated with diplacin we were able to study how the effect of both flickering and continuous light is reflected in the RNA content of the cytoplasm of the ganglion cells.

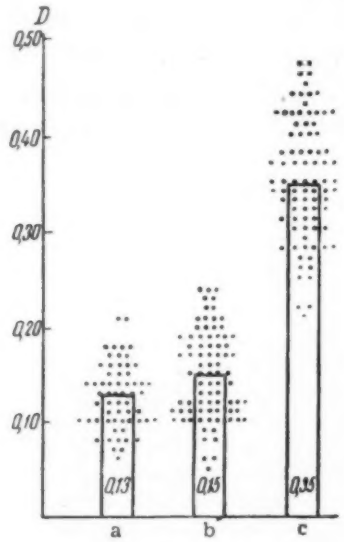
The first group of animals was left in the dark, the second illuminated with continuous light (~100 lx on the retina) and the third with flickering light delivered from a rotating disk with cut-out segments interrupting the light beam (frequency of flicker 2·5 c/s, equal periods of dark and light). The eyes were fixed 1 hr after the start of illumination and the ultra-violet method of cytophotometry used for the quantitative assay of the RNA in the cytoplasm of the ganglion cells. The method of treatment

* Biofizika **5:** No. 6, 749–750, 1960.

and analysis has been described earlier [4, 8, 9]. The cell RNA content in each experimental variant was defined as the average of measurements of 60–80 separate celles.

The results are presented diagrammatically in the figure.

The individual dots in each column show the optical density of RNA in the cytoplasm of one ganglion cell. Since, on light stimulation the dimensions of the ganglion cells are constant, the changes in the optical density of the RNA are proportional to the changes in the amount of this substance. Even without special statistical treatment the results of the experiments are quite plain. The amount of RNA in the cytoplasm of the ganglion cells showed virtually no change on constant illumination as compared with that in the dark. On excitation with interrupted light, the amount of RNA more than doubled.




Optical density of RNA (D) in ganglion cells of retina in various conditions of illumination. Time of illumination, 1 hr. Columns (and numerals) indicate mean values; dots, density of RNA in individual cells (thickness of sections 5$\mu$); measurements at $\lambda = 265$ m$\mu$. (a) darkness; (b) constant light; (c) flickering light.

Thus, the increase in RNA in the cytoplasm of the retinal ganglion cells always coincided with the state of excitation recorded electrophysiologically.

Hence, the change in RNA level can actually be taken as an indication (true, only an overall one) of the degree of cell activity. It is clear that ultra-violet cytophotometry can be used with success in disclosing the activity of the cells of the retina and other nerve formations, access to which is difficult even in microelectrode investigations.

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# BIOLOGICAL SIGNIFICANCE OF THE SOUNDS OF CERTAIN BLACK SEA FISH*

V. R. PROTASOV and M. I. ARONOV

Severtsov Institute of Animal Morphology, U.S.S.R.
Academy of Sciences, Moscow and the Sevastopol Kovalevskii Biological Station

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IN recent years the sounds of fishes [1] have been widely studied in connexion with problems of underwater acoustics, netless fishing and commercial prospecting. However, very little work has been devoted to an explanation of the biological significance of this phenomenon [2, 3].

The object of our investigation was to discover the significance of sounds emitted by certain Black Sea fish. The main experiments were carried out on the most intensively studied fish, *Corvina umbra* and several species of drum fish. To get the fullest picture we recorded the sounds of the grey mullet, red mullet and rock and sea bass. The experiments were performed in 1958–1959 in the aquaria and experimental ponds of the Karadag Biological Station of the Ukrainian S.S.R. Academy of Sciences. A hydrophone of the VNIRO system connected through an amplifier to a MAG-8 tape recorder was used to record the sounds.

The sound signals recorded can be divided into specific and unspecific. The first include signals of a definite form typical of an individual species of fish. This category includes the knocking of *Corvina umbra* — usually three successive blows — and the drum beat of *Crenilabrus griseus*. The unspecific group includes the sounds common, to differing degrees, to various species. These sounds are incidental to various other acts such as feeding, respiration, movement, fighting, etc. (munching, crunching, squeaking, cluttering, rustling, crackling, etc.).

An analysis was made of the most marked specific sound signals — the knocking of *Corvina umbra* and the drum beat of *Crenilabrus griseus* and also the unspecific but strong sounds of cluttering usually emitted by drum fish when feeding, the frequency characteristic of which has been described [4, 5].

*Corvina umbra*. The sounds were recorded in various conditions with simultaneous observation of the behaviour of the fish. It was established that this species emits characteristic sound signals when in pairs or groups but not when alone. They most often made sounds during abrupt movements, during and after turns and in slowing down. The knocking of these fish were repeatedly recorded on returning to the shoal after an unsuccessful attack on a moving prey — friar or young grey mullet. Our findings rule out the assumption that their sounds are secondary and involuntary appearing on movement. The fish which had earlier emitted sounds were stimulated with electric shocks (20 V and higher on connecting one of the poles of the battery to the dorsal fin) and mechanical blows. This treatment elicited sharp motor responses but no specific sounds apart from the hydrodynamic noises during the jerks of the fish.

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The specimens investigated were then placed with others of the same species and after a certain time lapse they again started to emit characteristic sounds. This indicates that such sounds are not "mechanical" secondary ones but are of a voluntary nature. In addition, cases were noted in which this species produced sounds when in a group and whilst not making strong movements or turns.

To investigate the role of the sounds of these fish as communication signals between individuals their knocking was recorded on tape and reproduced in the water by means of insulated earphones. No distinct reactions to these sounds were noted. Rarely, true, a fish approached the earphone in the same way as on delivery of signals from a sound generator, but as we were unable to control the frequency and strength of the signals delivered in the water, this method of reproduction had to be abandon ed. As a "source" of the sounds we later used the fish themselves and although this made the experiment dependent on the fish, it was possible to check on the situation in the aquarium by means of a hydrophone. The individual fish were isolated from one another in an aquarium by transparent and non-transparent partitions allowing penetration of sound (2–3 folds of gauze or a coarse meshed division). The fish were also paired in various combinations.

On isolating the fish from each other they did not knock in our experiments, but in the loudspeaker of the tape recorder weak sounds were heard resembling the ticking of a clock and crackling. In a number of cases on again pairing the fish the specific sounds were again heard. The sounds were usually made on pairing a male and female (as found after dissecting the fish) and less often for two males; a pair of females never produced the sounds. It should be noted that sexually-immature male yearlings also emitted the sounds.

The specific sounds of the male were very often recorded on eliciting a defence reaction by stimulations of moderate intensity, on movement of objects over the aquarium, when swimming next to others of the species or when a large fish of a different species swam close to them for example, *Trigla lucerna*. During these stimulations they raised the dorsal fin and executed a series of blows. For stronger stimuli — holding the fish, electric shocks, etc. — no sounds were emitted. It is significant that the sound made by the fish behind the partition on being disturbed by a moving object did not evoke any apparent reponse in the partner in the other division of the aquarium.

The experiments carried out on two blinded specimens and sighted fish in the dark did not reveal, in the range of frequencies recorded by our apparatus (50–10,000 c/s), any sounds made for purposes of location or orientation in the surroundings.

Thus, the specific sounds of *Corvina umbra* are from our findings characteristic of the males. Possibly, these sounds play a definite rôle in the interrelations of the sexes since according to published results [5] the sounds of this species are increased during the breeding season. These sounds most probably serve as threats — warning signals. It cannot be excluded that the knocking of this species in some cases serve as a call on approaching the group.

*Crenilabrus*. In the literature [4, 5] a description is given of fish emitting a drum beat, the species adherence of which has not been established. In our experiments it was seen that the drum beat is made by the *Crenilabrus griseus*. The fish is sonic in the following conditions.

When during feeding small *Crenalabrus ocellatus Forsk* pile up in the food corner, the *Crenilabrus griseus* on approaching the food with some delay drives away these fish and emits a strong drum beat. Such sounds were observed during encounters of the *Crenilabrus griseus* with *Crenilabrus tinca*, newly placed in the aquarium and of the same size, causing the fish to move away. The *Crenilabrus griseus* also emitted a drum beat on meeting relatively small prey — rock bass — which at this sound swam away. On placing *Corvina* (in size several times larger than the *Crenilabrus griseus*) in the experimental tank activity of the latter was suppressed. On encountering *Corvina*, the *Crenilabrus griseus* pressed itself to the bottom and did not make a sound.

It may thus be concluded that the drum beat acts as a warning or "threat" depending on the size of the object to which the signal is directed.

*Crenilabrus ocellatus and Crenilabrus tinca*. The unspecific clattering sound is strongly marked in drum fish of different species during feeding. The observations were made on a group of hungry *Crenilabrus ocellatus* and *Crenilabrus tinca* separated by opaque, sound passing partitions from several

feeding fish. The isolated fish became somewhat excited by the sounds behind the partition and started to swim vigorously around the aquarium. However, the reactions directed towards the source of the sound were not marked although the ability of fish to determine the direction from which a sound comes has been reported [6]. Only in individual cases when the experimental fish were less than 15–30 cm away from the partition was there directed movement towards the source of the sound. The fish also became active in reponse to the clattering sounds accompanying fighting between individuals. Against the background of increased motor activity elicited by the clattering, a visual stimulus provoked a searching or defence reaction of one of the fishes followed by a similar rapid reaction from the other fish.

Thus, this sound signal is not a specific one but it may be assumed that by raising the general activity, the sound acts as an "adjusting signal" enhancing visual orientation.

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OL.
5
960

# CHEMICAL STRUCTURE OF STREPTOMYCIN AND THE CHANGE IN FREQUENCY OF NUCLEAR DAMAGE OF IRRADIATED MAMMALIAN CELLS*

N. I. SHAPIRO and N. V. BELITSINA

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Moscow

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WE have earlier shown that streptomycin is an operative factor in changing the extent of radiation injury to mammalian cells [1, 2]. Streptomycin acts by changing the frequency of nuclear damage, i.e. by changing the probability of chromosome breaks. The modifying influence of streptomycin occurs after irradiation and depends on the concentration of the antibiotic; for some concentrations it reduces the radiation damage, others (higher) increase it. It has been established that the effectiveness of the preparation is limited to definite stages of the cell's mitotic cycle at the moment of treatment with streptomycin.

In the previous investigations, no examination was made of the relationship between the chemical structure of the antibiotic and the observed anti-radiation effect. Yet, an answer to the problem of whether there is within the complex chemical structure of streptomycin an active grouping responsible for the effect in question, and if so what is its structure, will undoubtedly bring us nearer to an understanding of the mechanism of repair of nuclear damage caused by ionizing radiations.

The present paper reports the first results of investigations along these lines. As is known, the streptomycin molecule consists of two parts; streptidine and streptobiosamine, each of which in iso-

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EFFECT OF VARIOUS CHEMICAL SUBSTANCES ON THE FREQUENCY OF CHROMOSOME ABERRATIONS IN IRRADIATED ERHLICH ASCITES CARCINOMA CELLS

| Experimental variant* | Streptomycin sulphate (1·0 mg/ml) | | Streptidine sulphate (0·3–0·5 mg/ml) | | Arginine hydrochloride (0·2 mg/ml) $NH_2-C(=NH)-NH-(CH_2)_3-CH(NH_2)-COOH$ | | Ornithine hydrochloride (0·16–0·23 mg/ml) $NH_2-(CH_2)_3-CH(NH_2)-COOH$ | | Norvaline (0·13–0·16 mg/ml) $CH_3-(CH_2)_3-CH(NH_2)-COOH$ | | Guanidine† (sulphate, hydrochloride, nitrate) (0·1–2·0mg/ml) $NH_2-C(=NH)-NH_2$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Test | Control | Test | Control | Test | Control | Test | Control | Test | Control | Test | Control |
| No. of repeat experiments | 15 | | 11 | | 7 | | 5 | | 4 | | 6 | |
| No. of anaphases studied | 1970 | 2102 | 2123 | 2200 | 1400 | 1400 | 800 | 800 | 800 | 800 | 800 | 800 |
| No. of anaphases with aberrations | 495 | 861 | 529 | 850 | 344 | 530 | 328 | 335 | 287 | 314 | 273 | 291 |
| % anaphases with aberrations | 25·1±1·0 | 40·9±1·1 | 24·9±0·9 | 38·6±1·0 | 24·6±1·1 | 37·8±1·3 | 41·0±1·7 | 41·9±1·7 | 35·9±1·7 | 39·2±1·7 | 34·1±1·7 | 36·4±1·7 |

*In some experiments with streptomycin, streptidine and arginine the substances were administered not after but before irradiation; since it was shown that the effectiveness of the action is the same for both modes of administration, these data have been summated in the table. All the substances studied as such, i.e. without irradiation did not change the frequency of chromosome disturbances.

†The effect of the sulphate, hydrochloride and nitrate of guanidine was investigated. The similarity in the results obtained allowed us to summate them.

VOL. 5 1960

lation does not possess antimicrobiol properties [3]. On inspecting the formula of streptomycin, the first thing to strike one is that the streptidine portion consists of two guanidino groups, which, as is known, in one form or another contribute to the chemical structure of a number of protective substances [4].

Accordingly, we first made a study of the effect of streptidine on the frequency of appearance of radiation-induced chromosome aberrations using as test object here, as in subsequent experiments, mouse Ehrlich ascites carcinoma cells. The work was carried out as follows: on the 7th day of development of the tumour, the ascitic fluid was extracted, heparin added and the whole exposed *in vitro* to 8000 r of $\gamma$-irradiation ($^{60}$Co) at a dose rate of 477 r/min. After irradiation, the ascitic fluid was diluted with saline to give a cell count of $7–10 \times 10^4$ per mm$^3$. The irradiated suspension was divided into two portions, to one of which streptidine (or one of the other substances investigated) was added. The substances were used in equimolar (in relation to streptomycin) or near equimolar concentrations. Intact mice were then inoculated intraperitoneally with 1·5–2·0 ml of the ascitic fluid. A control series with unirradiated suspensions was run on the same lines. After 24 hr the ascitic fluid was withdrawn and from it smears were prepared, fixed with methanol and stained with methylene blue (after Kölner and Stockinger). An estimate was made of the percentage of anaphases and early telophases with chromosome disturbances. The effect of streptidine on the frequency of nuclear damage is illustrated in the table.

OL.
5
960

The table gives for comparitive purposes the findings on the effect of streptomycin on the development of chromosome aberrations. The results point to the complete similarity of effect of the substances compared not only in relation to the nature of the effect, but also quantitatively. Thus, it may be concluded that the anti-radiation effect of streptomycin is wholly determined by the presence in its molecule of streptidine.

The further investigations concerned the nature of the effect of streptidine as stated, this substance contains reactive guanidino groups which, it may be assumed, are responsible to some extent for its beneficial influence. In this respect, the common amino acid — arginine — is similar in structure to that of streptidine and also contains a guanidino group in its molecule. True, in streptidine the guanidino group is linked with the 4-hydroxycyclohexane ring while in arginine it is attached to an amino acid (norvaline) residue. The results of a study of the effect of arginine on the development of nuclear damage are given in the table.

It may be concluded from this table that arginine is just as effective in combating the radiation effect as are streptomycin and streptidine. The complete similarity in the effect of these substances led to attempts at further analysis of the question as to which is the simplest compound responsible for the reversibility of the nuclear damage caused by irradiation. In this context, it was of particular interest to investigate the efficacy of substances similar in structure to the two constituents of arginine, namely guanidine, on the one hand, and such amino acids as norvaline or ornithine, on the other. The results of these investigations are also given in the table which shows that one of these substances exerted a favourable effect. Hence, from our experiments arginine is still the simplest substance responsible for reversibility of nuclear damage. In other words, this reverisbility is due to substances containing a guanidino group, but not to guanidine itself. Thus, to be effective the guanidino grouping must be linked with 4-hydroxycyclohexane in streptidine or with the amino acid residue of arginine. Guanidine alone or the amino acid without guanidine does not have this effect.

We are still not in a position to make any definite statement on the processes which lead to the reduction in chromosome aberrations. However, one cannot fail to point to their possible link with deoxyribonucleic acid (DNA). In a number of previous studies [5–7] it has been shown that on irradiation of DNA solution in the presence of oxygen an after-effect occurs. Thus, in an irradiated solution of DNA besides a fall in the viscosity during irradiation, there is a further fall even several hours after termination of irradiation. It has also been established that on irradiation in the absence of oxygen such an after-effect does not take place. Together with I. F. Nenarokova, we have obtained preliminary data indicating that on irradiation of suspensions of Ehrlich ascites carcinoma cells in anaerobic conditions, subsequent addition of streptomycin does not lead to a fall in the chromosome disturbances.

It may thus be supposed that irradiation of cancer cells gives rise to an after-effect similar in its course to that observed by a number of workers on irradiation of DNA solutions. It is interesting to note that glutathione when added to DNA solutions after irradiation completely prevents the after-effect [8]. Possibly, arginine exerts a similar influence on cancer cells.

Research now in progress is aimed at finding out both the nature of the processes of cell metabolism, interference with which leads to reduced nuclear damage in irradiated mammalian cells, and the mechanism of this effect.

In conclusion, the authors wish to express their deep gratitude to Doctors of Chemical Sciences, M. M. Botvinnik and A. S. Khokhlov for their valuable advice and to R. M. Zakirova for assistance in the work.

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VOL. 5 196

# DISCUSSION

## AN ERROR IN MEASURING THE INTENSITY OF PHOTOSYNTHESIS BY THE CONDUCTIMETRIC METHOD

V. L. VOZNESENSKII

Komarov Botanical Institute, U.S.S.R. Academy of Sciences, Leningrad

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RECENTLY many scientific institutes have been concerned with the application of a method of measuring the electrical conductivity of alkaline solutions absorbing $CO_2$ (conductimetry) in the determination of the intensity of plant photosynthesis. On the basis of this principle it is possible to design accurate, portable and simple instruments not requiring thermostats and fully suitable for work on the process of synthesis in the field.

In this note we should like to call the attention of investigators to a possible error in defining the intensity of photosynthesis introduced by use of over simplification of the measuring circuit.

The electrical measuring circuit is usually a more or less complicated Wheatstone bridge circuit,

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into one diagnonal of which is inserted a sound generator and in the other the amplifier with voltage indicator (indicator or telephones). To one arm of the bridge is connected a cell with the absorbing solution being measured (unknown resistance) and to another a certain standard resistance (wire or liquid). The other two resistances are a continuos wire resistance separated by an adjustable contact (rheostat). Such a system permits determination, from the position of the adjustable contact on the rheostat giving the minimum current (sound) in the voltage indicator, of the ratio of the resistance (electrical conductivity) of the test solution to that of the standard and from the value of this ratio and the standard the sought resistance is found.

In determining the intensity of photosynthesis usually an absorbent connected with the lamina chamber and an absorbent through which is passed a control sample of air are inserted in turn into the arm of the unknown resistance. Thus, the resistance (electrical conductivity) of both absorbing solutions is measured in relation to a standard and then on the basis of a previously plotted graph it is possible to obtain from these values the number of milligrams of $CO_2$ absorbed by the solutions in both test and control absorbents. From the difference in the volumes of $CO_2$ the intensity of photosynthesis of the plant used in the experiment can be computed.

In seeking to simplify such a measuring circuit some workers — Fridmann [1] and also Batyuk, Ribalka and Okanenko [2] — simultaneously include in the two arms of the bridge circuit measuring vessels connected to the test and control absorbents. The narrow band amplifier and output indicator and the original device for equalizing the reactive components of the resistances enabled the latter authors [2] to obtain good sensitivity of the entire apparatus (0·004 mg $CO_2$ per litre air). However, with the circuit used by them for connecting the absorbents they obtained measurements giving not "the difference in resistances of the cells of both absorbents", as the authors write, but the ratio of the resistances (electrical conductivities) of the solutions in the measuring cells of both absorbents or the ratio of the corresponding amounts of $CO_2$ absorbed by the test and control absorbent solutions. To compute the intensity of photosynthesis it is necessary to know not the ratio of these values but the difference between them. Now, as is known, a difference in values cannot be calculated unambiguously from a ratio of these values. It may be assumed that the authors made a preliminary calibration of the instruments (although they do not describe it) by passing known volumes of air through the control absorbent — with a definite $CO_2$ content (e.g. 0·03 per cent) and passing air with different concentrations of $CO_2$ through the test absorbent, the ratios of the electrical conductivities of the solutions were then measured. Knowing, for a definite intensity of photosynthesis, the volume of air passed through the absorbents and the ratio of the electrical conductivities of the solutions in both absorbents one can correctly calculate the intensity of photosynthesis of the test plant only if the concentration of the $CO_2$ in the ambient air is the same as during calibration of the instrument.

In normal conditions, as noted in several published findings [3–5, etc.], the $CO_2$ content of the air is never constant but shows daily and seasonal fluctuations dependent on the nature of the soil, the plant cover and many other factors. The mean concentration of atmospheric $CO_2$ is 0·03 per cent but its actual amount varies, according to the measurements of various authors, between a minimum of 0·013 per cent and maximum of 0·06–0·08 per cent. Comparing the values of the minimum and maximum concentrations of $CO_2$ from the data of each author with determinations of both values made separately by one investigator by the same method, we see that these values differ among themselves by 14–16 per cent and on average (from 11 determinations made by different workers) this value is 32 per cent. Okanenko [6], investigating the problem of fluctuations in the atmospheric $CO_2$ obtained data on the $CO_2$ content of different strata of air over a garden beet field. He concluded that depending on the meteorological conditions, the $CO_2$ content around garden beet leaves by noon is only about 70 per cent of that observed in the morning hours.

It is essential to take into consideration such considerable variations in atmospheric $CO_2$ when devising an instrument for measuring the intensity of photosynthesis. In confirmation of this we give the following examples.

We shall imagine that the control $K$ and test $O$ absorbents are inserted in the two arms of the measuring circuit and have resistances $R_1$ and $R_2$ (see Figure 1).

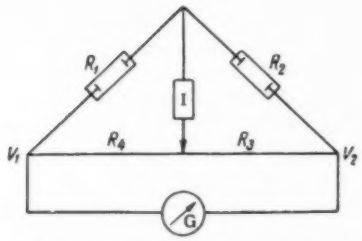


FIG. 1. Fundamental bridge circuit for incorporation of measuring cells of experimental and control absorbents on measurement of intensity of photosynthesis of plants. $R_1$ and $R_2$, resistances to be compared; $R_3$ and $R_4$, rheostat; I, a.c. generator G, galvanometer. Correct coupling: $R_1$, resistance of solution in test O or control $K$ absorbents; $R_2$, resistance of standard solution. Incorrect coupling: $R_1$, resistance of solution in test O absorbent; $R_2$, resistance of solution in control $K$ absorbent.

VOL. 5 1960

The circuit is supplied from an a.c. generator I with voltage $V$. Then the potential difference $V_1$–$V_2$ will be determined by the following relation (allowing only for the reactive components of the resistance)

$$V_1 - V_2 = \frac{R_1R_3 - R_2R_4}{(R_1 + R_2)(R_3 + R_4)} \cdot V. \tag{1}$$

The measurements themselves may be taken either by the null method or the method of the unbalanced bridge. With the null method, $V_1-V_2 = 0$ is possible only for $R_1R_3-R_2R_4 = 0$, hence the condition of balance of the bridge is $R_1/R_2 = R_4/R_3$.

We shall show that for a definite ratio of the values $R_1/R_2$ their difference $R_1-R_2$, determining the amount of $CO_2$ absorbed by the plant leaf, is an indeterminate value. We shall assume that $R_1 = 2r$ and $R_2 = r$; then $R_1/R_2 = 2$ and $R_1-R_2 = r$; for $R_1 = 4r$ and $R_2 = 2r$ again $R_1/R_2 = 2$ but $R_1-R_2 = 2r$, i.e. for the same ratio of the values, equal in this example to 2, the difference in the values determining the intensity of photosynthesis changes by a factor of two.

We shall also assume that measurements are taken by the method of unbalanced bridge and the galvanometer connected either directly into the circuit or at the output of the amplifier connected into the circuit. We shall show that in this case also for the same potential difference $V_1-V_2$, corresponding to the same deviation of the pointer, the difference in the resistances $R_1-R_2$ (and consequently the difference in the amounts of $CO_2$ absorbed by the control and test absorbents) may vary widely. For simplicity of argument, we assume that in equation (1) $R_3 = R_4$, then

$$V_1 - V_2 = \frac{R_1 - R_2}{2(R_1 + R_2)} V.$$

We shall take two cases:

(a) $R_1 = 2r$ and $R_2 = r$; substituting these values in the preceding equation, we get $V_1-V_2 = V/6$;

(b) Assuming $R_1 = 6r$ and $R_2 = 3r$ we again have $V_1-V_2 = V/6$, i.e. in both cases the potential difference obtained for $V_1-V_2$ (angle of deviation of pointer) is uniform although the difference in the values $R_1-R_2$ determining the intensity of photosynthesis being measured is not, as in the preceding example, uniform but varies by a factor of three.

Consequently, for a clear-cut definition of the difference in the values $R_1-R_2$ when measuring with a bridge circuit, it is necessary that one of these values be either constant and known or be determined from measurements of other parameters. Such parameters defining the resistance of the absorbing solution may be either a definite volume of air with constant $CO_2$ content or a definite time of passage of a current of air through the absorbent with constant concentration and rate of flow. But, in natural conditions, as stated above, the atmospheric $CO_2$ content fluctuates strongly and is

not a constant value. Therefore, in measuring the intensity of photosynthesis the value $R_1$ defined by the amount of $CO_2$ absorbed by the control solution will have different and unknown values if the determination is made with an unknown concentration of $CO_2$ and, consequently, the difference in the values $R_1 - R_2$ will also be an indefinite value.

We shall next take an example showing the influence exerted by change in the atmospheric $CO_2$ concentration on the calculated value of the intensity of photosynthesis. We shall assume that the instrument has been calibrated by passing 30 litres of gaseous mixture with a $CO_2$ concentration of 0·03 per cent (0·6 mg per litre air) through the control absorbent. In determining the intensity of photosynthesis 30 litres of air pass through each absorbent but with an initial $CO_2$ concentration of 0·025 per cent ($\sim$ 0·5 mg per litre air). The plant leaf absorbs an amount of $CO_2$ such that the ratio of the amounts of $CO_2$ absorbed by the solutions in the test and controls equal 1·25 per cent. (The ratio of the resistances of the control and test solutions measured on the bridge circuit will differ from this value and may be found on calibration of the instrument.) Since calibration is done for 0·03 per cent $CO_2$, then to calculate the amount absorbed by the plant leaf it is necessary to take the following steps; the control absorbent contains $30 \times 0{\cdot}6 = 18$ mg $CO_2$; the test $18/1{\cdot}25 = 14{\cdot}4$ mg $CO_2$ and plant leaf supposedly absorbs $18 - 14{\cdot}4 = 3{\cdot}6$ mg $CO_2$. In fact, the control contains $30 \times 0{\cdot}5 = 15$ mg and the test $15/1{\cdot}25 = 12$ mg $CO_2$ and, consequently, the amount absorbed by the plant is $15 - 12 = 3$ mg. As the example shows because the concentration of $CO_2$ in the air was taken as a constant value, the amount of $CO_2$ calculated as absorbed by the plant leaf — 3·6 mg — differs considerably from the actual amount — 3 mg. The error in calculation in this case is 20 per cent and is wholly determined by the deviation in the concentration of $CO_2$ adopted in calibration — 0·03 per cent — from that when determining the intensity of plant photosynthesis.

Such a level of error, for example in a study of the daily course of intensity of plant photosynthesis may lead to levelling out of the calculated values since in the morning, with an increased atmospheric $CO_2$ content, from calculations we arrive at low values, whereas during the day, with reduced concentrations, we conversely get increased values for the intensity of plant photosynthesis.

Consequently, the very principle of measuring the intensity of photosynthesis in field work proposed by some workers by determining the change in the ratio of electrical conductivities of test and control absorbents is not faithful and leads to errors in determining the intensity of plant photosynthesis. Moreover, this method does not allow one to obtain a value for the atmospheric content of $CO_2$ at the time of measuring the intensity of photosynthesis, which is an important factor in understanding the photosynthetic process.

From the investigations made by the Photosynthesis Laboratory of the Komarov Botanical Institute of the U.S.S.R. Academy of Sciences we conclude [7] that if we are not to fundamentally complicate the entire measuring circuit, the electrical conductivity of the absorbing solutions must be determined separately for test and control in relation to that of a standard present in a special cell. The use as standard resistance of an alkaline solution present in good thermal contact with the solutions in the test and control absorbents enables the investigator to avoid rigid thermostatting of the entire instrument. We then pass from the value of the electrical conductivity of the solutions by means of a calibration graph to the amount of $CO_2$ contained in the absorbing solutions and from the difference in these amounts in the test and control we can calculate the amount of carbon dioxide absorbed by the plant leaf in photosynthesis or given off in respiration.

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# CURRENT EVENTS

## PRIMARY AND INITIAL MECHANISMS OF BIOLOGICAL ACTION OF NUCLEAR RADIATIONS

VOL. 5 196

In April 1960, an All-Union Symposium on the primary and initial mechanisms of the effect of ionizing radiations was held in Moscow. The first part of the programme was devoted to discussion of the primary radiation processes at molecular level. A. G. Pasynskii examined radiation-induced chemical changes in biopolymers and pointed to the fundamental difference between them and similar changes in low molecular weight substances. In complex macromolecules, the probability of internal transformations is heightened, e.g. irradiated amino acids display a far larger number of maxima in the e.p.r. spectra than the proteins apparently, the electron expelled from the protein molecule is rapidly replaced by an electron of the cysteine radical). This mechanism prevents rupture of the C-C and C-H bonds and thereby radiation damage to the macromolecule is lessened. The low temperature phosphorescent spectra of egg and serum albumins do not contain the tyrosine component which is distinctly in evidence in equivalent mixtures of amino acids. Under the action of OH radicals, SH groups of cysteine irradiated *in vacuo* are readily oxidized whereas the SH groups of ovalbumin are not oxidized even after a dose of $6 \times 10^5$ r. In the presence of 5 molar guanidine, oxidation of the SH groups of the protein in solution occurs in the same way as for cysteine solution.

In solutions of guanidine the hydrogen bonds of the protein molecule are ruptured. The participation of the SH groups in the network of hydrogen bonds leads to lowering of the reactivity of the protein molecule. Apparently rupture of these bonds by guanidine raises the mobility of the hydrogen of the SH group.

Comparison of the number of SH groups oxidized in dry egg protein irradiated *in vacuo* with a dose of $5 \times 10^6$ r and the free radicals (e.p.r. spectrum) has shown that the number of oxidized SH groups is 60–100 times greater than the number of radicals. It thus follows that the presence of the SH groups in the irradiated protein molecule is not connected with radical formation. On the other hand, the oxidized SH groups ($9 \times 10^{18}$/g protein) is in precise quantitative agreement with the number of ionizations occurring for this dose in the protein ($I = 1$). This concordance can be explained on the basis of the polarization waves formed around the ionization centre causing re-orientation of the dipoles and rupture of about 12–14 H bonds. Quantitative agreement is found with the number of H bonds ruptured on thermal denaturation of protein and under the influence of guanidin on protein irradiated in solution.

The mechanism of electron transfer or rupture of H bonds is of general significance for the structural changes in biopolymers. In the living cell the biopolymers form part of the molecular structures determining as it were the second level of radiation specificity of the cell. Damage to isolated macromolecule in homogenous solution cannot reflect the specific after effects which appear in radiation damage to the macromolecule of the intracellular structure.

Such after effects may consist of changes in the permeability of the intracellular membranes with

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subsequent disturbances in the processes of cell metabolism. This point can be illustrated by the following experiment. It has been shown that in polymer films the radiation "lacing together" of macromolecules causes disturbances measuring in size some tens of molecules in the structure of the film. The formation of "lacings" in DNP monolayers leads to increase in the area from 0·26 to 0·36 m²/mg which must lead to increase in the permeability of the monolayer. In a complex suspension of peroxidase and ribonucleoprotein, each enzyme particle was surrounded by 5–8 layers of nucleoprotein separating the enzyme from the substrate — ascorbic acid. On irradiation of such a system with doses causing damage to the layers of nucleoprotein but not affecting the enzyme or substrate, the rate of the enzymatic reaction was increased by 40–50 per cent. Change in the steady state of an irradiated cell may be related to change in the conditions of the intracellular interaction between enzymes and substrate. The structural disintegration of the irradiated cell is of great importance in the subsequent development of radiation damage to the entire organism although the initial damage may be localized in structures of molecular dimensions.

V. K. Tkach referred to the opposite effect of radiations on monolayers. For 24–26 hr a sharply marked radiation after effect expressed in considerable packing of the monolayer is noted; its thickness is reduced from 20 to 8Å. The polymer molecules pass from the horizontal to a vertical position (*sic*).

A. L. Shabadash confirmed the ideas put forward on the radiation change in intracellular septa which is of fundamental importance in the development of radiation damage. Cytochemical investigations have established the constancy of enzyme and substrate in the irradiated cell, whereas the structural formations of the cell, for example, the mitochondria undergo radiation injuries leading to changes in the conditions of the biochemical interactions within the cell.

L. A. Tummerman emphasized the radiobiological importance of the semiconductor conductivity of protein structures. In the production of radiation damage to the cell it is necessary to take accoune of the rupture of weak bonds as an end link in the process of energy migration.

In his report A. M. Kuzin reviewed the various mechanisms by which ionizing radiations influenc DNA synthesis: (1) change in the pre-existing DNA as matrices for synthesis; (2) disturbance of oxidative phosphorylation in the nucleus which is essential for the formation of polyphosphorus derivatives of the deoxynucleotides; (3) disturbance in the reducing processes responsible for conversion of ribose into deoxyribose nucleotides; and (4) disturbance in methylation during formation of thymidine from uridyl nucleotides.

The investigations of Kuzin, Struchkov and Strazhevskaya showed the high radiosensitivity of high polymer DNA (Molecular weight $6–8 \times 10^6$). In dilute DNA solutions a change in viscosity is observed after only 100–500 r, whereas DNA extracted from irradiated tissue in the absence of the action of nuclease retains its stability.

The investigations of V. I. Tokarskaya have confirmed her previously published findings of change in the quality of DNA synthesized in plants after irradiation. She detected a sharp reduction in the incorporation of thymidine into the DNA molecule 24 hr after irradiation, whereas the rate of incorporation of the purine bases into the newly synthesized DNA was almost as before. Emphasis was placed on the possible importance of synthesis of qualitatively different DNA in explaining the late after effects of radiation.

A. M. Kuzin compared the results obtained in his laboratory with those of Okada (1960) who showed in *in vitro* experiments sharp retardation in the incorporation of thymidine into irradiated DNA of animal cells but with unchanged uptake of cytidine. The speaker concluded that the arrest of DNA synthesis after low radiation doses can hardly be the cause of the change in the matrix properties of DNA molecules. The change in the quality of the newly synthesized DNA after irradiation is apparently the consequence of disturbed synthesis of the polyphosphorus derivatives of thymidine or change in the DNA structure as a matrix.

M. N. Meisel' stressed the great radiosensitivity of phosphorylation processes in the nuclei. The change in the nuclear composition of DNA in the cells of radiosensitive organs must have far-reaching implications in relation to synthesis of RNA and proteins.

S. Ye. Manoilov considered that change in the metabolic processes of nucleic acids is a secondary phenomenon which leads to cell mutations. The synthesis of DNA requires an enzyme system which can only function in the presence of a definite ratio of nucleotides.

V. I. Tkach underlined the great importance of low doses in the process of complexing ions and proteins.

Ia. L. Shekhtman drew attention to the great similarity between the DNA and RNA molecules. The problem arises of the high radiosensitivity of DNA present in the cell nucleus, apparently in a special physico-chemical state due to structuring with water. In the view of Shekhtman, the direct effect of radiation is of prime importance in *in vivo* experiments.

The second session of the symposium considered the primary radiobiological processes at the cellular level.

M. N. Meisel' emphasized that analysis of radiation damage to the cell can be made both by elucidation of the patterns of disturbance of the cell functional structures and by examination of the sequence of their recovery.

In the cell there are at least three interrelated systems of functional structures with the character of "controlling systems": nucleus, mitochondria and ergastoplasm. The degree of radiosensitivity of these systems varies, but they are all subject to radiation damage both directly and mediated. The structure of the cytoplasm exerts a decisive influence on the realization of the potential damage to the nuclear structures and their reparation.

The speaker drew general conclusions from the findings on the basic disturbances detected in the functional state of the nuclear and cytoplasmatic structures of the irradiated cell. The initial damage to the nuclei, the more severe the higher the radiation dose, is characterized by the following disturbances: liberation of potassium ions, depression of phosphorylation of mononucleotides, temporary delay in nuclear division, inhibition of DNA synthesis and formation and secretion into the cytoplasm of DPN coenzymes. These disturbances are accompanied by physico-chemical changes in the nuclear nucleoproteins, they become labile with changes in primary (ultra-violet) and secondary (capacity for binding with acridines) fluorescence.

The initial damage to the cytoplasm is primarily linked with disturbance in the physico-chemical properties of the membrane structures accompanied by anomalous redistribution of potassium, sodium and phsophorus ions. These disturbances are intensified during active cell metabolism. In all cells — from micro-organisms to those of higher organisms — depression of oxidative phosphorylation is observed with disturbed carbohydrate metabolism (partial switch to synthesis of fats and lipoids inhibition of glycolysis). It is possible that one of the reasons for these changes is depression of DPN coenzyme synthesis in the nuclei and disturbed supply to the mitochondria and structural elements of the ergastoplasm.

The speaker demonstrated schemes illustrating the functional interrelation between the main cell structures in normal and irradiated cells.

Kondatrev taking lymphocytes as his example showed that radiation damage to the cell nucleus disappears 30 min to 6 hr after irradiation. The subsequent changes are found in the cytoplasm.

N. I. Shapiro showed that primary radiation injury is not a single factor but a composite of explicit and latent changes in the structures. Irradiation of dry seeds is accompanied by an oxygen effect which may be removed by sodium metabisulphite. This substance not only removes the intensifying effect of oxygen in relation to radiation damage, but also reduces the chromosome aberrations due to irradiation.

N. V. Timofeev-Resovskii also pointed to a series of substances which lessen the oxygen effect; alanine, cystine, etc. If 30 min after the combined effect of alanine and oxygen, the seeds are washed and oxygen passed, the number of chromosome aberrations rises. It may thus be concluded that the protective action of alanine is manifest only in relation to oxygen.

The joint effect of oxygen and raised temperature (53°) enhances radiation damage to cells: their effects are summated.

A. L. Shabadash postulated the reversibility of chromosome aberrations in cells in the resting stage, this applies to the majority of cells of the body. Worthy of particular note are those of the central

nervous system which on irradiation of the animal with sublethal doses show changes in the physico-chemical properties of the intracellular structures within only 10 min of irradiation. The isoelectric point of the individual intracellular formations shifts by one unit of pH. These changes are caused by doses which correspond to the stimulation thresholds of the cells.

K. S. Trincher proposed that the term radiolability be introduced to characterize the reactivity of the cell. Cell radiolability is a value inversely proportional to the minimum dose of penetrating radiation which causes reversible changes in the morpho-physiological structure of the cell. The term radiosensitivity should be employed in determinations of irreversible damage to the cell. This is a value inversely proportional to the minimum dose causing irreversible morpho-physiological changes of the cell. Thus, the radiolability of nerve cells, for example, or their ability to react reversibly to the radiation stimulus considerably exceeds their radiosensitivity.

The third part of the symposium heard a report by N. N. Demin on early radiation induced biochemical changes in whole body systems.

In whole body conditions simultaneous changes occur both at molecular and cellular level. It is therefore difficult to differentiate the primary from the secondary reactions and the results of the damaging effect of ionizing radiations from the closely associated compensatory and reparative processes.

In whole body conditions disturbances in the content and metabolism of nucleic substances, lipids and low molecular substances with biological activity and appreciable changes in oxidative phosphorylation are manifest early. Special attention needs to be paid not so much to the changes in individual groups of substances but to the reactions of complexes, systems of conversions and disturbances at the level of the microstructures. In this respect, of exceptional interest are the shifts in the lipo- and mucoprotein structures which are connected with the conditions of permeability, the functional properties of microstructures and the interaction of these complexes with biologically-active agents on radiation injury to the organism.

The primary and early reactions of the body to irradiation are very complex in view of the diversity of influences of the radiation factor. On whole body irradiation one has to reckon with the simultaneous effect of radiation on neuroreceptors and the tissues of the nervous system, endocrine organs and of other parts of the injured body. It is necessary to ascertain experimentally the importance of the individual pathways of the radiation effect. A solution has not been found to the problem of nervous influences, hormonal effects, direct changes in tissue metabolism and so-called toxaemia. There is no doubt that later, at various stages of development of radiation sickness disturbances in the functions of individual systems are revealed as the dominant factor.

In whole body conditions it is particularly important to discover which of the early biochemical changes are an expression of catabolism and destruction and which are compensatory-reparative ones. It is necessary to establish experimentally which of these changes are actually decisive in the development of subsequent stages of radiation sickness.

D. E. Grodzenskii drew attention to the early biochemical reactions of radiation pathology; synthesis of cholesterol and higher fatty acids is accelerated by 15–20 times in the liver and suprarenals.

P. G. Durmishyan referred to the early starting up of processes in the body on which depend both attenuation and intensification of the reparative processes. Acetylcholine 3 min after irradiation affects the rate of conduction of stimuli. There is early appearance of intensified functioning of the suprazonal system. In the suprarenals the amount of ascorbic acid diminishes.

N. N. Federova noted the changes in nucleic acids and proteins as primary reactions of the body to irradiation. Desoxycytidine is excreted in the urine at different times after irradiation with sublethal doses. In the body abnormal proteins appear, detected by changes in the optical properties of the tissues.

S. Ye. Manoilov considered that the initial reaction on radiation damage to the body is connected with changes in proteins containing metals. Catalase and cytochromes pass into the oxidized form.

L. Kh. Eidus noted a correlation between the radiobiological effect and the appearance of unpaired electrons in protein molecules due to the effect of radiation. Later under the influence of heat or oxygen the energy is realized as radiation damage to the molecule.

V. K. Tkach drew attention to the property of proteins to form complexes with ions, which may be investigated by the method of electron paramagnetic resonance. The sorption properties of the proteins are influenced by the state of the central nervous sysetm and the age of the animal. On irradiation, the sorption properties of proteins change.

P. I. Tseitlin reported on his experiments with DNA solutions. At radiation doses of $10^3$ r a decline in viscosity was observed without change in molecular weight. At comparatively low doses overpacking of the DNA molecule occurs in the form of compression. If the DNA is compressed by acidification of the medium (pH 2·7) the DNA acquires high resistance.

K. S. Trincher referred to changes in the radiosensitivity of erythrocytes on addition of serum proteins suspended in saline. The high radioresistance of red cells in whole blood is due to the protective action of the water-soluble proteins. In their absence the erythrocyte is a radiosensitive cell, to which the radiation damage from a dose of 500 r may be detected by the method of isotonic alkaline haemolysis.

Kerkes investigated the effect of low doses on the nuclear structures. In the guinea-pig irradiation with a dose of 4 r gives a 10-fold increase in chromosome transformations.

Ye. F. Romantsev observed excretion of desoxycytidine in the urine on irradiation of animals with doses of 100–400 r, its amount increasing with the dose. Hence, the presence of a chain mechanism of the reaction may be assumed.

The symposium mapped out the lines of future research on the problems outlined above.

K. S. TRINCHER
V. I. TOKARSKAYA

VOL.
5
196

## REVIEW

J. BUREŠ, M. PETRÁŇ and J. ZACHAR. Electrophysiological Methods is Biological Research. Published by the Czechoslovak Academy of Sciences. Prague, 1960*

THIS book by the well-known Czech electrophysiologists J. Bures, M. Petráň (Prague) and J. Zachar (Bratislava), recently published in the English language, is devoted to the theory and practice of electrophysiological research over a wide biological field and is an exceptionally valuable contribution to the development of physiology and biophysics.

This outstanding book contains the following chapters: I — Electrophysiological phenomena — theoretical basis; II — Electrophysiological apparatus and techniques; III — General electrophysiology of cells and tissues; IV — Electrophysiology of isolated, excitable structures *in vitro*; V — Electrophysiology of peripheral excitable structures *in situ*; VI — Electrophysiology of receptors; VII — Electrophysiology of the spinal cord; VIII — Electrophysiology of the cortex of the large hemispheres; and IX — Electrophysiology of subcortical structures.

In addition to these main chapters, there is also a valuable appendix in the form of "stereotoxic atlases" for cats, rabbits and rats (compiled by members of the staff of the Anatomical Institute of the Mechanical Faculty of Karlovy University — E. Fifkova and I. Marshal).

Mere enumeration of the chapters of the book shows that it deals with the main lines of work in the field of modern electrophysiology. The authors have successfully solved the intricate problem of outlining in a most succinct yet full manner the theoretical problems of modern electroph ysiology together with a very thoughtful and detailed account of the techniques of the main electrophysiological experiments. This book is at the same time a guide to the extensive practicum of electrophysiology

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for under-graduates and post-graduates and to the everyday work in research laboratories in the sphere of electrophysiology and is a handy reference work for specialists.

With the wealth of recent literature on electrophysiology (including reference books and methodological works) it would have seemed difficult to produce anything original and comprehensive. Yet, our Czech colleagues have written a quite original and very useful book. Although not my intension to give a detailed review of the book, I should like to stress the exceptional interest of the pages in the first chapter devoted to discussion of the problems of electrochemistry, closely linked with the theory and practice of electrophysiology. I feel that a very valuable and useful contribution to further work is provided by the detailed account of the theory and practice of modern methods of recording bioelectrical phenomena, the detailed description of different types of stimulators, amplifiers and recording devices and also the technique of preparing macro- and micro-electrodes of various types. There can be no doubt that the attention of biophysicists and comparative physiologists will be attracted by the third chapter outlining the theory and methods of study of electrophysiological phenomena in plants, developing embryos and single cells. And finally, absorbing reading matter is provided by more than two hundred pages on the electrophysiology of the peripheral and central nervous system, reflecting not only the rich experience of world electrophysiology, but the original and very valuable contribution of the authors themselves.

OL.
5
960

Of course, the book does not go into every existing special technique nor deal particular with all objects of electrophysiological research: the reviewer has, for example, noted omission of a description of the technique of recording the biopotentials from the stretch receptors of Crustacea and unicellular animal organisms (Infusoria). Other readers will probably note omissions of the same nature. Yet, this does not diminish the high level and particularly useful nature of the book.

A very favourable impression is left by the bibliography listing over six hundred publications which enhances the book as a reference work. The Soviet reader cannot fail to note that the book reflects to a degree unparalleled in foreign publications the scientific and methodological achievements of the scientists of our country.

In conclusion, one cannot but express deep satisfaction and a sense of pleasure at the scientific progress made by the strong team of young electrophysiologists of Chechoslovakia, who within a short space of time have been able to establish centres of modern electrophysiology in Prague and Bratislava which are gaining in international stature. Finally, it is to be hoped that a Russian translation of this very useful book will appear at the earliest possible moment. There can be no doubt that it will become not only a hand-book for Soviet specialists and biophysicists but an important manual for wide usage in the universities in preparing new scientific cadres in one of the most important branches of modern science.

KH. S. KOSHTOYANTS